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Chapter 1

General introduction

History and background

Topical hemostatic agents have been used for thousands of years to stop bleeding. The ancient Egyptians applied fresh meat to a wound on the first day as a hemostatic agent. In the following days, treatment was continued with grease, herbs and honey [1]. In ancient Greece, wounds inflicted on the battlefield, were treated with herbs that were also described to have analgesic effects. Unfortunately, the used plants were not documented and still remain unknown [2]. Native Americans are thought to have used scrapings from the inside of fresh animal hides mixed with hot sand and downy eagle feathers as topical hemostatic agent [3]. Nowadays, many topical hemostatic agents are available to the surgeons, dentists and ER personnel. These agents include gauze sponges, absorbable gelatin sponges, absorbable collagen sponges, oxidized cellulose, chitin dressing, platelet concentrates and fibrin sealants. Gelatin, collagen and cellulose hemostatic agents are among the most widely used absorbable agents since several decades and will be described in more detail [4]. More recently, gelatin and collagen are increasingly combined with topical thrombin as this is the final enzyme of the clotting cascade [5].

Gelatin

Gelatin, a porous form of denatured collagen, was introduced as a topical hemostatic agent in 1945 [6]. Gelatin foam is manufactured from animal skin gelatin whipped and baked into its sponge form [4]. It provides a physical matrix in which a clot can form while absorbing surrounding fluids [7]. The large surface area of the numerous interstices may attract and damage platelets in the blood which enters the sponge, thus, liberating thromboplastin [8]. The material can be applied as a film, sponge or powder. According to the literature, it is important to note that gelatin sponges swell more than collagen products and can cause compressive complications, especially when used near nerves or in confined spaces [9]. Although gelatin is derived from animal products, it is largely nonantigenic. Absorption of the gelatin foam is completed within 4 to 6 weeks [4].

Collagen

Microfibrillar type I collagen was developed as a topical hemostatic agent by Hait in 1970. It is derived from bovine corium and is available in powder form, a nonwoven sheet and as a sponge [9]. When the collagen gets into contact with blood, platelets adhere to the collagen fibrils and become activated [10]. The activated platelets change their shape from discoid to spherical and release procoagulant factors like adenosine diphosphate (ADP) and thromboxane A_2 [11]. This activation leads to platelet aggregation and thrombus formation [12]. Hemostasis is usually achieved within 2 to 5 minutes. Since its mechanism of action depends on platelet activation, it is less effective in patients with severe thrombocytopenia, but suc-

cessfully achieves hemostasis even after large doses of heparin [13]. Some swelling occurs, although this is less than the swelling of gelatin [4]. Collagen is only mildly immunoreactive and degradation of the material is completed in 6 to 12 weeks [14].

Cellulose

Oxidized cellulose was introduced in 1942, wheras oxidized regenerated cellulose was developed in 1960 and is manufactured from wood pulp, which contains about 50% cellulose by mass. In order to obtain purified cellulose, it has to be decomposed and than recomposed into regenerated cellulose [4]. Cellulose acts as a physical matrix for clot formation, although the exact mechanism of its action is unknown but probably depends on surface interactions with proteins and platelets [4,15]. The material also seems to confer hemostasis by decreasing the pH and acting as a caustic agent leading to hemostasis by generating an artificial clot [16]. The cellulose hemostatic agent can absorb up to 7 times its own weight and its greatest use has been for the control of oozing from broad surfaces [17,18]. Although the low pH exerts an antimicrobial effect, it might also increase inflammation of surrounding tissue and delay wound healing [19]. Because of this risk of foreign body reactions, it is recommended that the smallest possible amount of cellulose be used and that it be removed once hemostasis is achieved [9]. It may take up to 6 weeks to fully degrade, but its presence has been detected as late as 1 year when used in cardiac surgery [9,20].

Possible antigenicity and pathogen transmission of collagen and thrombin

Injectable soluble collagen was approved by the US Food and Drug Administration (FDA) in 1981, to be used as a subcutaneous implant to correct minor dermatological defects. Soon after its widespread use, questions about its antigenicity were raised [21,22]. This caused concern in the medical community over the use of collagen in general. Approximately 3% of patients developed a hypersensitivity reaction to the bovine collagen implant during a standard forearm skin test [23]. Another report indicated that an additional 5% of the patients whose screening test were initially negative will have serum antibodies to collagen [24]. In 1991 another problem arose in Europe when it was found that cattle were dying from a syndrome known as bovine spongiform encephalitis (BSE). The infectious agent that causes BSE disease was found to be a prion (a proteinatious particle) that also causes scrapie in sheep and goats. As this infectious agent apparently crossed the species barrier, concern was raised over the use of bovine products, especially those used in medical products [14]. Although there is no report of human BSE-infection from collagen-based medical devices, the raw material of the collagen should not come from a country with known BSE cases [19].
The antigenicity of topical bovine thrombin has also raised concerns because of the potential for antibodies directed against the product [5]. The development of antibodies directed against bovine coagulation factors like thrombin and clotting factor V has been reported in

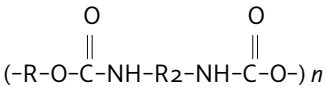

Figure 1. General formula of polyurethane.

patients treated with various bovine thrombin preparations [25]. The resulting antibodies can cross-react with human endogenous coagulation proteins and might lead to coagulopathies like severe bleeding or thrombosis [26-31]. Because of these safety concerns, recombinant human thrombin was developed to provide an alternative to bovine thrombin. Recombinant human thrombin has been shown to be considerably less immunogenic than bovine thrombin while the efficacy is similar [5].

Although the clinical impact of antigenicity and potential pathogen transmission is low up till now, a fully synthetic biodegradable hemostatic agent would prevent the potential risk of blood-borne pathogen transmission and immunization. Furthermore, the production process of a synthetic material allows greater control over material properties and tissue responses, which gives a synthetic material another advantage over animal-derived products [32]. The animal-derived hemostatic agents are relatively cheap materials. Therefore, a new synthetic topical hemostatic agent should preferably be made of a material that can be produced at the same low cost.

Polyurethane

A synthetic material that has been investigated extensively is biodegradable polyurethane (PU). Its possible hemostatic properties were recognized by Van Minnen who studied the biological behaviour of PU and its applications in dentoalveolar surgery [33]. Polyurethane is a polymer in which the repeating unit contains a urethane moiety. Urethane linkages are formed by the reaction of isocyanates (-N=C=O) with hydroxyl (-O-H)-functional molecules like polyol [34]. In this reaction the hydrogen atom of the hydroxyl group is transferred to the nitrogen atom of the isocyanate [35]. The general formula for polyurethane is shown in figure 1 [36].

Segmented polyurethanes have been used as biomaterials for several decades due to their biocompatibility and favorable physical properties like strength and flexibility [37].
These first polyurethanes were intended for use as long-term implant materials such as pacemaker lead insulators [38]. After years of production of PUs, manufacturers found them susceptible to degradation [36]. Therefore, a lot of research has been performed to elucidate the mechanisms of degradation and increase the biostability of PU [39-41]. In contrast, the development of intentionally degradable biomedical polyurethanes has received relatively

little interest [42]. The main reason for this lack of interest was due to the use of the toxic aromatic diisocyanate precursors (e.g. toluene diisocyanate) which may lead to carcinogenic compounds when the polyurethane is degraded [43]. The development of non-toxic aliphatic amino acid-derived diisocyanates has removed the obstacle to synthesizing degradable polyurethanes [44,45]. This has led to the development of biomedical PUs that are designed to biodegrade and remodel to form functional tissue [34]. The most common approach to the synthesis of these degradable polyurethanes has involved the use of polyester soft segments like polyhydroxyacids and poly-ε-caprolactone [46-48]. By varying the molecular structure, the rate of hydrolysis and therewith the degradability could be controlled [49]. The combination of hard and soft segments provides the polyurethane with its unique physical properties, such as flexibility in combination with mechanical strength [50].
The degradability of PU can be further increased by combining it with polyethylene oxide (PEO) [42,51]. This is attributed to the hydrophilic nature of PEO because the presence of a greater volume of water within the polyurethane can allow for a greater amount of hydrolysis, and thus degradation, to take place [52].

The polyurethane described in this thesis has uniform hard segments composed of butanediol and 1,4-butanediisocyanate and soft segments of DL-lactide and ε-caprolactone. The hard segments had a uniform length of five urethane moieties. The good biocompatibility of a comparable PU has been demonstrated in an *in vivo* study [53]. In this study the PU samples were resorbed almost completely after three years. A more rapid degradation period would be favorable to take away the risk of secondary inflammation. To increase the degradability, polyethylene glycol (PEG) which chemical properties are identical to PEO, was added to the material. The details of the composition and the synthesis of the polyurethanes investigated in this thesis are described in the Materials & Methods sections of Chapters 2 to 6.

Anticoagulant therapy

The use and need for topical hemostatic agents might increase with the aging population of Western society. As cardiac diseases and the subsequent risk of thrombo-embolism are more frequent among the elderly, an increasing number of patients might be considered for anticoagulant therapy [54]. These patients show a tendency towards higher bleeding risk and might benefit from hemostatic agents, during acute surgical procedures or after traumatic injuries [55].
With respect to anticoagulant therapy, special attention has to be paid to intraoral surgical procedures. At the Academic Centre for Dentistry Amsterdam (ACTA) in the Netherlands, a guideline has been developed that recommends continuation of anticoagulant therapy during dentoalveolar surgery under well-described conditions [56]. The potentially higher bleeding risk is accepted because discontinuation of anticoagulant therapy can result in thromboembolic events that may have far more severe consequences than limited postoperative

bleeding [57]. The guideline [56] was developed by gathering the best available evidence on this subject in the literature. The practical consequences in the Dutch setting have not been investigated.
The antifibrinolytic agent tranexamic acid has an important role in this guideline. This agent works by competitively inhibiting the activation of plasminogen, reducing the formation of plasmin, which is responsible for the degradation of fibrin clots [58]. Although typically administered intravenously, tranexamic acid has been used topically to control bleeding in a variety of specialities [59]. It significantly reduced mean blood losses after oral surgery in patients with haemophilia and was effective as a mouthwash in dental patients receiving oral anticoagulants [60-62].

Aims of this thesis

The aim of the present thesis was threefold:

1. To assess the hemostatic efficacy of polyurethane foam with polyethylene glycol and its working mechanism.
2. To analyse the time to complete degradation and biocompatibility of polyurethane foam with polyethylene glycol.
3. To evaluate the risk of bleeding incidents after dentoalveolar surgery in patients on oral anticoagulant therapy.

The hemostatic efficacy of PU was compared to commercially available hemostatic agents *in vitro* (**chapter 2**), *in vivo* (**chapter 3**) and in a rat tail-tip study (**chapter 4**). The hemostatic action and mechanical properties of PU were analysed and compared to gelatin and collagen in **chapter 5**.
In **chapter 6**, the degradation process and biocompatibility of PU in rats and rabbits is described and compared to gelatin and collagen.
The bleeding incidence after dentoalveolar surgery in patients with and without anticoagulant therapy was evaluated in **chapter 7**.
Finally, an integrated discussion of these studies is given in **chapter 8**.

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Chapter 2

In vitro analysis of polyurethane foam as a topical hemostatic agent

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Abstract

Topical hemostatic agents can be used to treat problematic bleedings in patients who undergo surgery. Widely used are the collagen- and gelatin-based hemostats. This study aimed to develop a fully synthetic, biodegradable hemostatic agent to avoid exposure to animal antigens. In this *in vitro* study the suitability of different newly developed polyurethane-based foams as a hemostatic agent has been evaluated and compared to commonly used agents.
An experimental *in vitro* test model was used in which human blood flowed through the test material. Different modified polyurethane foams were compared to collagen and gelatin.
The best coagulation was achieved with collagen. The results of the polyurethane foam improved significantly by increasing the amount of polyethylene glycol.
Therefore, the increase of the polyethylene glycol concentration seems a promising approach. Additional *in vivo* studies will have to be implemented to assess the application of polyurethane foam as a topical hemostatic agent.

Introduction

Topical hemostatic agents can be used to treat problematic bleedings in patients who undergo surgery. The benefits of local hemostats include improving blood conservation by reducing blood loss, shortening the time of hemostasis, avoiding the adverse effects of systemic hemostatic drugs and saving transfusion blood. Adverse effects can be foreign body reaction, infection, and granuloma formation [1]. Different types of hemostatic agents are available, each with their own characteristics. The following characteristics should be considered in search of an ideal hemostatic material: ease of application and removal, bioresorption potential, suturability, antigenicity, and tissue reactivity effects [2]. The possibility to adjust absorbability and flexibility of the material would be a further advantage [3]. Some of the most widely used topical hemostatic agents nowadays are collagen and gelatin which are animal-derived products. Procoagulant substances like thrombin or fibrin are frequently added to these agents to increase their hemostatic efficacy [1].

However, the use of products from animal or human origin has the potential risk of blood-borne pathogens and immunization. Examples are Bovine Spongiform Encephalopathy (BSE) and Creutzfeldt-Jakob disease. Bovine collagen intended for collagen-based medical devices should therefore not come from a country with known BSE cases [1]. The use of bovine-derived components in hemostats has resulted in a number of reports of immunization to bovine thrombin. The resulting antibodies can cross-react with blood clotting factor V and lead to coagulopathy [4].

A fully synthetic, biodegradable material with hemostatic properties would prevent these potential risks. Polyurethane (PU) with uniform hard segments composed of butanediol and 1,4-butanediisocyanate and soft segments of DL-lactide, ε-caprolactone and polyethylene glycol (PEG) is a synthetic biodegradable material with possible hemostatic properties. Coagulation is initiated upon exposure to a PU surface while the PEG makes the material more hydrophilic which increases the attraction of platelets [5]. In a previous *in vivo* study the good biocompatibility of a comparable PU without PEG moieties has been demonstrated [6].

It is not known whether PU foam has hemostatic properties or can be modified to a hemostatic agent. We questioned in this *in vitro* study if PU foam can achieve similar hemostatic properties as commercially available products. Therefore, PU foams were compared to an absorbable collagen hemostat (Novacol) and an absorbable gelatin sponge (Spongostan). Different modifications were applied to the PU foam to test whether they might increase its hemostatic efficacy. The PU foam was enriched with rFVIIa (recombinant factor VIIa), phospholipids, ADP (adenosine diphosphate) and thrombin. Factor VII is one of the central proteins in the coagulation cascade. When factor VIIa forms a complex with tissue factor (TF) the extrinsic pathway of the coagulation cascade is initiated [7]. Phospholipids greatly enhance the binding of factor VIIa to TF. ADP interacts with platelets, leading to further platelet activation [8]. Thrombin is a coagulation protein that has many effects in the coagulation cascade including platelet activation and the conversion of fibrinogen into fibrin [9]. Furthermore,

some of the PU foams were treated with 'plasma glow discharge' which creates a more hydrophilic foam with enhanced platelet binding and activation [10].
An experimental *in vitro* test model was used in which fresh human whole blood flowed through the test materials. This test model was based on the Thrombostat 4000® which is used to measure *in vitro* bleeding time and volume [11].

Materials

In this study tests have been performed with PU, Novacol (absorbable collagen hemostat; Bioprof BV, Moerkapelle, the Netherlands) and Spongostan (absorbable gelatin sponge; Johnson & Johnson, Skipton, UK).
The used PU is a block-copolymer composed of urethane hard segments and co-polyether-ester soft segments. This was done to achieve a more rapid degradation. The soft segments (total molecular weight: 2.000 g/mol) consisted of 50% DL-lactide and 50% ε-caprolactone. In a first PU foam formulation, PEG 1.000 was used as initiator for the soft segments synthesis. In a second PU formulation, PEG 20.000 was added after the synthesis in a mass ratio of 3 (first PU formulation) to 1 (PEG 20.000) to prepare a blend. The urethane segments were synthesized with 1,4-butanediisocyanate (BDI) and 1,4-butanediol (BDO). They had a uniform length of 5 urethane moieties, which resulted in a PU with BDI-BDO-BDI-BDO-BDI urethane segments in the polymer. The PU was then dissolved in 1,4-dioxane. After dissolving, the solution was poured into a mold and cooled down to −18ºC. The solution was freeze-dried at 3 mbar to remove the 1,4-dioxane crystals, resulting in an highly porous foam with a porosity of 97% and an overall PEG-content of 40 wt%. Overall porosity was calculated after determining the weight and dimensions of the foams.
The second PU foam formulation created a much more hydrophilic foam due to the increased PEG concentration. This foam had a porosity of 97% and an overall PEG-content of 55 wt%. The foams were processed to round slices with a diameter of 4 mm and a thickness of 2 mm. The foams were ultimately sterilized using ethylene oxide (EtO). The polymers and foams were manufactured by Polyganics BV (Groningen, the Netherlands). The collagen and gelatin materials were, like the PU foams, processed to round slices with a diameter of 4 mm and a thickness of 2 mm.
After the sterilisation process 145 foams were modified to potentially increase their hemostatic efficacy. This was done by adding 10 µl of a procoagulant substance or surface treatment. In table 1 an overview is presented of the added procoagulant substances and surface treatment.

Procoagulant substances

Prior to adding the substances, the PU foams were treated for 15 minutes with 5 µl of a 15% glutaraldehyde solution to increase the attachment of the substances. Treatment with glu-

Table 1. Overview of the added substances to the PU foams.

PEG concentration of PU foam (Wt%)	Added substance or treatment	Number of treated foams
40	Recombinant FVIIa (0.1 mg/ml)	30
40	Phospholipid solution (100 mg/ml)	20
40	Adenosine diphosphate (ADP) (0.085 mg/ml)	20
40	Bovine thrombin (100 NIH U/ml)	10
55	Recombinant FVIIa (0.1 mg/ml)	25
40	Saline (0.9% NaCl)	10
40	Plasma glow discharge	30

taraldehyde is a procedure which can be employed to stabilize the binding of enzymes [12]. The excess of glutaraldehyde was washed away with copious amounts of phosphate buffer solution (pH 7.4) and ultimately with ultra-pure water. The substances were immediately applied to the foams one hour before testing using a volumetric pipette.

Surface treatment

Glow discharge treatment was performed at 50 W during 5 minutes at both sides. Vacuum was achieved by a dual trap vacuum pump and was always lower than 1800 Pa prior to argon (99.996%, Hoekloos Nederland B.V., the Netherlands) inlet. The argon pressure during plasma treatment was 500 Pa. After glow discharge, the reactor chamber was ventilated with ambient air. The surface characterisation of the treated PU foams was analysed by X-ray photoemission spectroscopy. This showed an increase of oxygen and nitrogen which resulted in a lower contact angle and thus higher hydrophilicity.

Methods

Blood preparation

The study was approved by the ethics committee of the University Medical Center Groningen, the Netherlands. The blood that was used for this study was obtained from 10 healthy adult volunteers. Exclusion criteria were a known disease or use of drugs that could have influence on blood coagulation. Each volunteer donated 30 ml of blood which was tested for normal trombocyte count. To prevent the blood from instant clotting, heparin was added to a concentration of 1.5 IU of heparin per ml blood. The blood was then divided over 30 microcentrifuge tubes, one ml per tube and kept at room temperature. This way 30 tests could be performed from each blood donation, the tests were finished within two hours after the blood was obtained. Each session the materials were tested in a different order to level the influence of time after the blood donation. A series of tests was performed without material

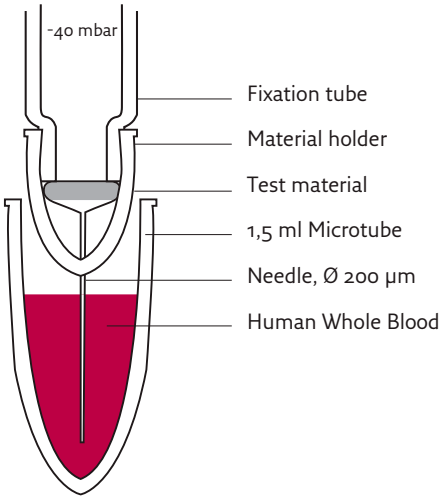

Figure 1. In vitro test model. Suction of human whole blood was performed with a constant pressure of -40 mbar. Ø, diameter.

as a control group. Recombinant tissue factor (rTF) in a concentration of 5 pM was added to each ml of blood to resemble the wound situation *in vivo*. This was necessary because no detectible concentrations of active TF are present in the blood of healthy donors [13]. While in a wound situation, extravascular TF is exposed to the blood and binds plasma factor VIIa [7].

Test device

An experimental *in vitro* test model was used which was based on the Thrombostat 4000® (Von der Goltz, Seeon, Germany). This device was used because of the possibility to insert different test materials in an existing model for measuring hemostasis *in vitro*. In this model blood flowed through the fixated test materials with a constant pressure of -40 mbar. Suction of the blood was performed through a needle with a diameter of 200 µm to create shear stress (Figure 1). The shear stress was necessary to mimic capillary bleeding which is an important platelet activator *in vivo*. The Thrombostat calculated the amount of blood flow through the test material over a period of 90 seconds. As a derivative for the extent of coagulation the blood flow deceleration was determined. This was calculated out of the blood volume that had passed the test material after 30, 60 and 90 seconds. The volume after 30 seconds was used to obtain the velocity of blood flow at 30 seconds. This was done by dividing the volume after 30 seconds by 30 and defined as initial velocity (V_i) in µl/s. The final velocity (V_f) in µl/s was calculated by dividing the volume that had flowed between 60 and 90 seconds by 30. The deceleration (d) in µl/s^2 could be calculated by dividing the difference between initial and final velocity by the time interval in seconds (t) using the formula $d = (V_f - V_i)/t$.

Table 2. Mean deceleration and standard deviation of the different materials.

Material	Mean deceleration (µl/s²)	Standard deviation
Collagen	0.036	0.022
Gelatin	0.021	0.016
PU (55 wt% PEG)	0.015	0.007
PU (55 wt% PEG) + rFVIIa	0.015	0.006
PU + glow discharge	0.012	0.011
PU + thrombin	0.009	0.011
PU + saline	0.008	0.005
PU (40 wt% PEG) + rFVIIa	0.004	0.011
PU (40 wt% PEG)	0.002	0.010
No material	-0.001	0.009
PU + ADP	-0.002	0.015
PU + PL	-0.003	0.015

PU, polyurethane; PEG, polyethylene glycol; rFVIIa, recombinant factor VIIa; ADP, adenosine diphosphate; PL, phospholipids.

Thrombin-antithrombin complexes (TAT)

The extent of thrombin generation *in vitro* was determined by measuring thrombin-antithrombin complexes (TAT) in two of the blood samples. The measurement was performed 30 minutes after venous blood collection. In two of the blood samples that had flowed through the PU foam (55 wt% PEG) with and without rFVIIa the amount of TAT was also determined. After the experiment 500 µl of blood was collected and added to 50 µl EDTA-solution (0.1 mol/ml) to prevent further generation of thrombin. The blood sample was then centrifuged, and 200 µl of the supernatants were collected and stored at -20ºC until further analysis. Enzyme-linked immunosorbent assay (Cedarlane Ltd., Burlington, Ont., Canada) was employed for measurement of the TAT complexes.

Statistical Analysis

Statistical analysis was performed using SPSS 16.0. Group differences were calculated by the Mann-Whitney-U-test. P values less than 0.05 were considered statistically significant.

Results

The mean decelerations and their standard deviations were calculated per material and are presented in order of efficacy in table 2.
For a clear overview the mean decelerations are also presented in a boxplot (Figure 2).

Table 3. Thrombin-antithrombin complexes in the different blood samples.

Blood sample	TAT (mg/L)	Standard Deviation
Before testing	1.38	0.06
After PU	1.36	0.07
After PU + rFVIIa	2.40	0.12

PU, polyurethane; rFVIIa, recombinant factor VIIa.

The best results were achieved with collagen which performed significantly better than the other materials ($p<0.001$). The second best results were achieved with gelatin which performed significantly better than the control group without material and than the PU foams with 40 wt% PEG, with or without rFVIIa ($p<0.001$). The PU foams with 40 wt% PEG showed no significant difference compared to the control group without material. The PU foams with 55 wt% PEG showed a significantly higher deceleration than the PU foams with 40 wt% PEG ($p<0.02$). Again the addition of rFVIIa to the foams gave no improvement of the results. No significant difference was observed between the PU foams with 55 wt% PEG and gelatin.
The results of the modified PU foams (40 wt% PEG) are also presented in a boxplot (Figure 3).

In the group with the modified PU foams, collagen again performed significantly better than the other materials ($p<0.001$). The PU foams which were treated with 'plasma glow discharge' showed a significantly higher deceleration than the tests without material ($p<0.02$). The PU foams which were enriched with phospholipids, adenosine diphosphate, saline and thrombin showed no significantly different results from the tests without material.
The blood took an average time of 12.1 seconds (SD: 0.83) to reach the material after suction was started. When the blood took more than 14 seconds to reach the material the results from these tests were excluded. This was the case in 23 out of the 308 tests.

Thrombin-antithrombin complexes (TAT)

Three duplo measurements were performed to assess the amount of TAT complexes before and after flow through the PU foam (55 wt% PEG) with and without rFVIIa. Results of this measurement are presented in table 3.

The plasma concentration of TAT complexes *in vitro* 30 minutes after venous blood collection is above the normal range (1.0 - 4.1µg/L) *in vivo* [14]. Approximately the same concentration of TAT was found in the blood samples that had flowed through the PU foam (55 wt% PEG) without rFVIIa. The blood samples that had flowed through the PU foam (55 wt% PEG) with rFVIIa showed a higher concentration of TAT complexes.

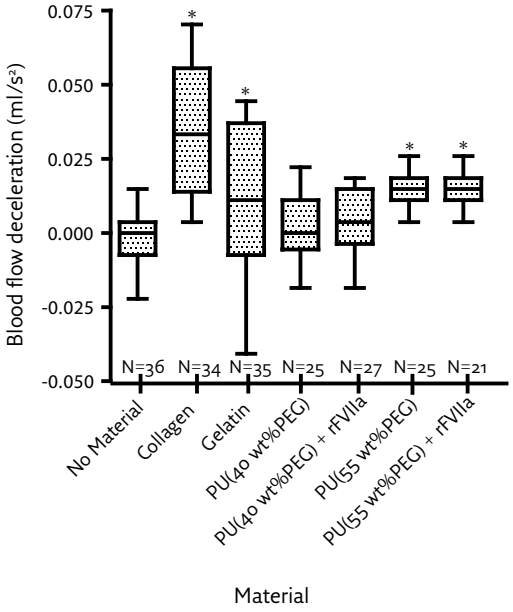

Figure 2. Schematic representation of decelerations for the different test materials. The number of tested samples (n) is noted. PU, polyurethane; PEG, polyethylene glycol; rFVIIa, recombinant factor VIIa. For each group, the line in the middle of the box represents the median. The lower and the upper edges of the box are the 1st and 3rd quartile, respectively. The fences are drawn to the nearest value not exceeding 1.5 (interquartile range). *P<0.001 compared with control group (No Material).

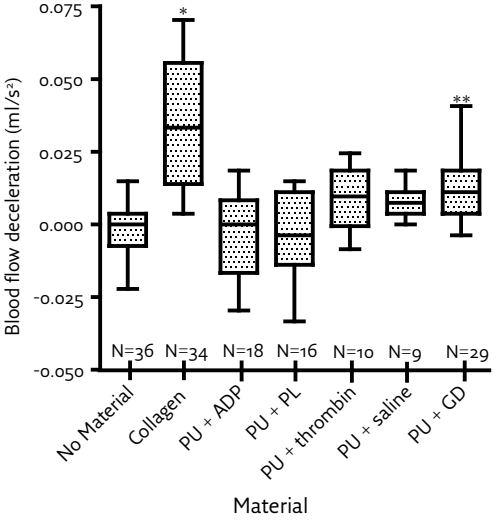

Figure 3. Schematic representation of decelerations for the different modified PU foams (40 wt% PEG). The number of tested samples (n) is noted. The results of collagen have been added to this boxplot as a reference. PU, polyurethane; PL, phospholipids; ADP, adenosine diphosphate; GD, glow discharge. For each group, the line in the middle of the box represents the median. The lower and the upper edges of the box are the 1st and 3rd quartile, respectively. The fences are drawn to the nearest value not exceeding 1.5 (interquartile range). *P<0.001 compared with control group (No Material). **P<0.05 compared with control group (No Material).

Discussion

An experimental *in vitro* test model was used to compare the synthetic PU foam to commercially available hemostatic materials. Additional rTF was added to the blood to more closely resemble the wound situation *in vivo*. Different modifications have been applied to the PU foam to increase its hemostatic efficacy. The addition of rFVIIa to the PU foam did not influence the results. The effect of intravenous rFVIIa has been proven to reduce blood loss in severe blunt trauma patients in a multicenter randomized controlled trial [15]. In a study in rats the local use of a microporous polysaccharide hemosphere (MPH) in combination with freeze-dried rFVIIa significantly improved hemostasis [16]. The failure of rFVIIa to improve the results of PU foam in this study could indicate that the extrinsic pathway of the coagulation cascade was not adequately activated in the current test model. The extrinsic pathway is initiated by the combination of TF and FVII and clotting time with normal protein concentrations is approximately 8.6 seconds [17]. In the used test model the blood flowed through the material during 90 seconds. Under the influence of heparin which inhibits thrombin and factor Xa this time period might have been too short [18]. Another explanation could be that the applied rFVIIa was flushed out of the PU foam by the blood and therefore did not contribute to the coagulation. The same could be true for the other applied substances which gave no improved blood coagulation. Another explanation is that the complexity of the coagulation cascade might not be adequately resembled *in vitro*. This process was further complicated by the necessary addition of heparin which inhibits the coagulation cascade [18].

The PU foams which have been enriched with phospholipids, adenosine diphosphate and thrombin gave no improved results. With the addition of thrombin to a local hemostatic agent good results have been achieved in *in vivo* studies [19,20].

The good results of collagen in this study could be explained by its action mechanism which is based predominantly on platelet aggregation. Collagen-based hemostats attract platelets when they get in contact with blood. The platelets adhere to collagen fibrils and degranulate, thereby triggering platelet aggregation [21].

The improved results of PU foam after modification of the PEG concentration could indicate that this enhanced the attraction of platelets. In a study by Lee et al. [22] the platelet adhesion to a PU surface increased slightly when short PEG chains were added whereas long PEG chains prevented platelet adhesion. The absorbable properties of PEG might contribute to its hemostatic effect by concentration of the endogenous coagulation factors and platelets. The effect of cellulose- and polysaccharide-based hemostats is based partly on this mechanism [17,23].

To analyse the influence of adding fluids to the foam some PU foams have been enriched with saline. As expected these foams gave no improved results compared to the PU foam without modifications.

The treatment of PU foams with glow discharge led to significantly better results. This should be contributed to the electrostatic charge at the membrane surface which creates a more

hydrophilic material. By increasing the hydrophilicity of polyethylene Spijker et al. [10] found more platelet adhesion and activation of the clotting system. In 23 out of the 308 tests the blood took more than 14 seconds to reach the material and therefore the results from these tests were excluded. The delay in blood flow could be due to obstruction of the 200 µm needle.

An advantage of our *in vitro* model was the easy way to test the efficacy of a large number of modifications in multifold experiments with human blood. A few drawbacks of the *in vitro* model were also demonstrated. Generation of thrombin after collection of blood can lead to activation of platelets and subsequent loss of response to agonists [24]. The addition of heparin to the blood does not completely block the generation of thrombin *in vitro* [25]. This is supported by the results of the TAT generation test. The TAT concentration in blood before contact with any hemostatic agent showed a 1000-fold increase compared to normal blood. The lowering blood temperature might also have contributed to a diminished platelet function [26].

Conclusions

In this *in vitro* coagulation study the best results were achieved with collagen. Increase of the PEG concentration improved the results of PU foam significantly and seems a promising approach. There were no significant differences between this modified polyurethane foam and gelatin. The results of collagen however, could not be matched. Additional *in vivo* studies will have to be implemented to assess the application of polyurethane foam as a local hemostatic agent.

Acknowledgements

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Chapter 3

In vivo hemostatic efficacy of polyurethane foam compared to collagen and gelatin

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Abstract

Topical hemostatic agents are used in all surgical disciplines. Most of these hemostats are based on animal derived products like collagen and gelatin. They carry the potential risk of pathogen transmission. A newly developed biodegradable, fully synthetic hemostatic agent based on polyurethane foam (PU) with 55 wt% polyethylene glycol (PEG) would prevent these potential risks.

The hemostatic efficacy of this new agent was compared to gelatin and collagen in humans who underwent extraction of an upper and lower molar (split-mouth model). After extraction of a molar in the maxilla and mandible a PU foam and collagen or gelatin was inserted in the extraction socket for two minutes. Hereafter the agents were removed and stored in EDTA to stop coagulation. Then the concentration of coagulation parameters thrombin-antithrombin III (TAT) complexes, fibrinogen and thromboxane B_2 (TxB_2) in blood extracts from the agents was measured. The concentrations were also determined in baseline blood samples which were collected from the extraction socket.

The concentrations of TAT and TxB_2 were significantly increased and fibrinogen concentration was significantly reduced compared to baseline wound blood concentrations indicating enhanced hemostasis. No significant differences were seen in the concentrations of these coagulation parameters in the three different hemostatic agents.

These results show that PU combined with 55 wt% PEG is a promising alternative for the animal derived hemostatic agents.

Introduction

Topical hemostatic agents are used in all surgical disciplines. Most of the mechanical hemostats are based on animal derived products like collagen and gelatin. Therefore, they carry the potential risk of pathogen transmission which can result in diseases like Creutzfeld-Jacob or Bovine Spongiform Encephalopathy (BSE) [1].

A newly developed fully synthetic hemostatic agent would avoid these potential risks. Furthermore, the production process of a synthetic material allows greater control over material properties and tissue responses, which gives a synthetic material another advantage over animal derived products [2]. The animal derived hemostatic agents are relatively cheap materials. Therefore, a new synthetic topical hemostatic agent should preferably be made of a material that can be produced at the same low cost.

A blend of modified polyurethane foam (PU) and polyethylene glycol (PEG) was processed to a synthetic, biodegradable material with possible hemostatic properties. The biodegradability and biocompatibility of a comparable PU has been demonstrated in a previous study [3]. In an animal study in which this PU foam was used to close oroantral communications, complete bony regeneration was seen after one year with only small PU fragments at a microscopic level [4]. This was a PU with 5 wt% PEG, whereas our PU with 55 wt% PEG will be resorbed much faster. The synthetic PU foam can be produced at low cost but the development of bioresorbable products has become expensive due to laws and regulations. Therewith, it can be expected that the PU will be more expensive than the collagen- and gelatin-based hemostatic agents.

When a biomaterial like PU comes into contact with blood, adsorption of factor XII initiates the contact activation pathway of the coagulation cascade and leads to platelet adhesion and activation [5]. The hemostatic efficacy of this material was improved by increasing the concentration of PEG up to 55 wt% as shown by *in vitro* study [6]. Hemostasis, however, is complex and difficult to resemble *in vitro* [7]. Therefore, an *in vivo* study was performed in which the polyurethane foam was compared with a commercially available collagen- and gelatin-based hemostatic agent. The purpose of this study was to investigate if the modified PU could achieve similar results as the collagen- and gelatin-based hemostatic agents. The hemostatic efficacy of the materials was analysed by measurement of blood coagulation parameters. Thrombin-Antithrombin III (TAT) complexes, fibrinogen and thromboxane B_2 (TxB_2) were measured in blood which was derived out of the materials after use in a human wound. The concentration of TAT complexes is accepted as an index of thrombin generation *in vivo* and thus a measurement for the amount of coagulation [8]. Fibrinogen is converted into fibrin by thrombin [9]. Reduction of fibrinogen is therefore also an indicator of the amount of coagulation. Thromboxane B_2 is an inactive metabolite of thromboxane A_2 which can be used to quantify platelet aggregation [10].

Materials and Methods

Subjects

The study population consisted of sixty patients who were referred to the Department of Oral and Maxillofacial Surgery of the University Hospital Groningen, the Netherlands, for extraction of an upper and lower molar. The molars were extracted because of insufficient space in the dental arch or because they were difficult to clean for the patient. None of the patients had an active pericoronitis or periodontal disease during the time of extraction. The patients were otherwise healthy and none of them used any medication. The study protocol was approved by the local ethical committee and conducted according to the guidelines for good clinical practice and the declaration of Helsinki. Written informed consent was obtained from each patient before inclusion.

Materials

In this study PU foam was compared to a collagen and gelatin hemostatic agent. The used PU was a block-copolymer composed of urethane hard segments and co-polyether-ester soft segments. The soft segments (total molecular weight: 2.000 g/mol) consisted of 50% DL-lactide and 50% ε-caprolactone. PEG 1.000 was used as initiator for the soft segments synthesis, and after that PEG 20.000 was added in a mass ratio of 3 (first PU formulation) to 1 (PEG 20.000) to prepare a blend. The urethane segments were synthesized with 1,4-butanediisocyanate (BDI) and 1,4-butanediol (BDO). They had a uniform length of 5 urethane moieties, which resulted in a PU with BDI-BDO-BDI-BDO-BDI urethane segments in the polymer. The PU was then dissolved in 1,4-dioxane. After dissolving, the solution was poured into a mold and cooled down to −18ºC. The solution was freeze-dried at 3 mbar to remove the 1,4-dioxane crystals, resulting in an highly porous foam with a porosity of 97% and an overall PEG-content of 55 wt%. Overall porosity was calculated after determining the weight and dimensions of the foams. The foams had a cylindrical shape with a size of 1 cm^3 and were ultimately sterilized using ethylene oxide. The polymers and foams were manufactured by Polyganics BV (Groningen, the Netherlands).

For the collagen hemostat, Hémocollagène (absorbable collagen hemostat; Septodont, Saint-Maur-des-Fossés, France) was used with a size of 1 cm^3. For the gelatin hemostat, Spongostan (absorbable gelatin sponge; Johnson&Johnson, Skipton, United Kingdom) was used with a size of 1 cm^3.

Study protocol

We designed an experimental, prospective, randomized, split mouth protocol in which PU foam, collagen and gelatin were tested. Randomisation was generated with a computer random number generator. PU foam was tested in all patients and compared to a collagen or gelatin sponge in random order. After extraction of the lower molar, 0.2 ml of blood was collected from the extraction socket with a syringe. The blood was stored in a cup with 200

µl of 0.2 M ethylenediaminetetraacetic acid (EDTA) solution to chelate calcium and prevent further activation of coagulation proteins [11].
Hereafter, one of the test materials was inserted in the alveolus during two minutes. After extraction of the upper molar another test material was inserted in this alveolus for two minutes. Continuous suction was performed around the alveolus to ensure that saliva did not flow in the alveolus. After the two minutes had passed, each material was stored in a cup filled with EDTA solution. The amount of EDTA solution was adjusted for each test material to match the mean amount of blood that was absorbed by each material. In the cups for the PU foams 250 µl of EDTA solution was inserted, for the collagen sponge 170 µl and for the gelatin sponge 160 µl. Thus, the ratio of blood:EDTA solution was kept at 1:1.
The cups with blood and materials were centrifuged at 13.000 revolutions per minute (rpm) during 1 minute in a MSE Micro Centaur. This causes the plasma, cellular release products and EDTA solution to poor out of the foams. The aliquots of plasma were collected and stored at -80ºC. The samples were thawed just before analysis.

TAT complex immunoassays

The plasma samples were diluted 1 in 1000 in dilution buffer (0.1% BSA in PBS pH 7.4). Hereafter, the samples were assayed for TAT complexes using a commercially available immunoassay kit (Enzygnost TAT micro; Behring Diagnostics, Westwood, MA). This assay is a sandwich enzyme linked immunosorbent assay (ELISA).

Fibrinogen immunoassays

Thirty paired plasma samples were subjected to the detection of fibrinogen. The plasma samples were diluted 1 in 5000 in dilution buffer. Fibrinogen was measured with an ELISA (Enzyme Research Laboratories Ltd, South Bend, IN, USA).

Thromboxane B_2 immunoassays

TxB_2 generation was measured in plasma samples diluted 1 in 1000 in dilution buffer using immunoassay (Cayman Chemical, Ann Arbor, MI, USA) as indicated by the manufacturer's protocol.

Statistical analysis

Data were analyzed using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as mean and standard deviation (SD). The results for the group of 30 patients who received PU and gelatin were analysed separately from the group of 30 patients who received PU and collagen. The paired samples were analyzed with a Wilcoxon's signed rank test.
Linear regression analysis was used to determine the relationship between extraction time and baseline values. A two-sided p-value < 0.05 was considered statistically significant.

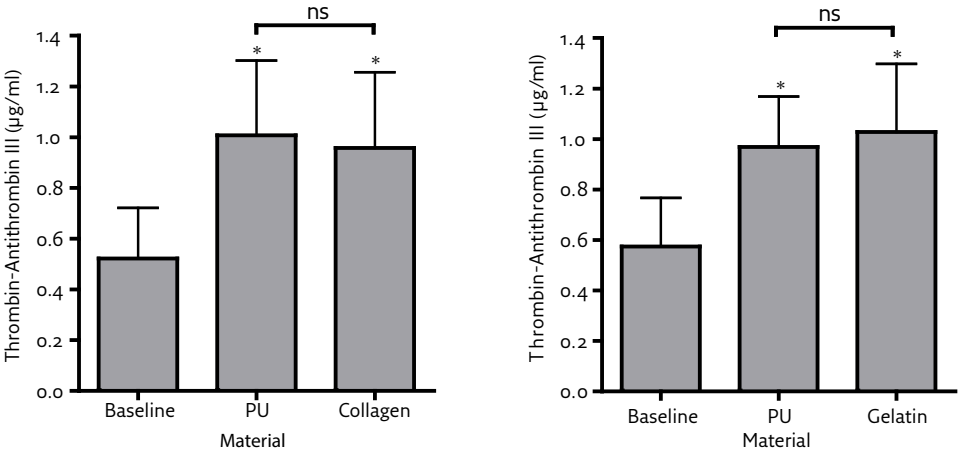

Figure 1. Mean (± SD) thrombin-antithrombin III concentration for the different test materials and baseline samples. PU: polyurethane foam, ns: not significant. * = significant compared to baseline.

Results

Sixty patients (33 male, 27 female) with a mean age (range) of 26 (19-47) were included in the study. To analyse the degree of coagulation in the different samples we measured the concentration of TAT complexes, fibrinogen and TxB_2. We found that the use of test materials significantly increased the concentration of TAT complexes and TxB_2 compared with baseline values. The fibrinogen was decreased significantly compared with baseline values. Moreover, we found that baseline values, obtained from wound blood, were significantly different from reported normal blood values. In this wound blood, TAT and TxB_2 were markedly increased and fibrinogen decreased.

The results were separately analysed for the two groups of 30 patients and are therefore shown in different bar charts. Figure 1 shows the TAT concentration for the different test materials and baseline samples.

The mean TAT concentrations for PU (1.01 ± 0.29 µg/ml) and collagen (0.96 ± 0.30 µg/ml) were higher than the baseline concentration of 0.52 ± 0.20 µg/ml ($p < 0.001$) in this group. Between PU and collagen there was no significant difference ($p = 0.13$).

The concentrations of TAT complexes in the group with PU and gelatin were 0.97 ± 0.20 µg/ml and 1.03 ± 0.27 µg/ml respectively. Therewith, the concentrations were significantly higher than the mean concentration of TAT complexes in the baseline samples which was 0.57 ± 0.19 µg/ml ($p < 0.001$). Between PU and gelatin there were no significant differences in concentration of TAT complexes ($p = 0.30$).

Figure 2 shows the results for the fibrinogen concentration of the test materials and baseline samples.

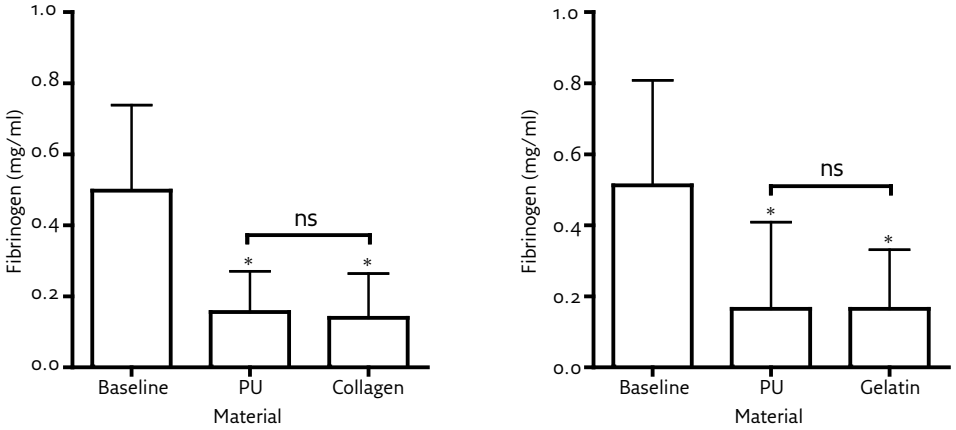

Figure 2. Mean (± SD) fibrinogen concentrations for the different test materials and baseline samples. PU: polyurethane foam, ns: not significant. * = significant compared to baseline.

The mean concentration of fibrinogen in PU (0.16 ± 0.11 mg/ml) and collagen (0.14 ± 0.12 mg/ml) was significantly lower than the mean concentration of fibrinogen in the baseline samples (0.50 ± 0.24 mg/ml) with a p value < 0.001 for this group. Between PU and collagen no significant differences were seen for the concentration of fibrinogen (p = 0.88).

In the other group the fibrinogen concentration was also significantly lower in PU (0.16 ± 0.24 mg/ml) and gelatin (0.16 ± 0.17 mg/ml) than in the baseline blood samples (0.51 ± 0.30 mg/ml) with a p-value < 0.001. The fibrinogen concentration was not significantly different between PU and gelatin (p = 0.49).

To investigate the influence of the test materials on platelet aggregation the TxB_2 concentration was measured. In figure 3 the data for the two groups is presented.

The mean concentration of TxB_2 in the first group was 28.5 ± 24.3 ng/ml for the PU and 20.3 ± 17.5 ng/ml for the collagen. This was significantly higher than the mean concentration of 8.5 ± 4.3 ng/ml in the baseline samples (p < 0.001). Between PU and collagen the concentration of TxB_2 was not significant (p = 0.16).

The mean concentration of TxB_2 in the second group was 31.9 ± 20.0 ng/ml for the PU and 28.8 ± 22.5 ng/ml for the gelatin. This was again significantly higher than the mean concentration of 7.7 ± 4.6 ng/ml in the baseline samples (p < 0.001). No significant differences were seen between the TxB_2 concentrations for PU and gelatin (p = 0.70).

The extraction time for the molars varied in the different subjects ranging from 2 to 15 minutes with a mean extraction time of 6.8 minutes. To investigate if the extraction time correlated with the baseline concentrations of TAT complexes, fibrinogen and TXB_2 a linear regression analysis was performed. This analysis revealed that the extraction time was not correlated with the baseline concentrations of TAT complexes, fibrinogen and TxB_2 (Table 1).

Table 1. Regression analysis of the correlation of extraction time with the baseline concentrations of TAT complexes, fibrinogen en TxB_2.

Coagulation parameter	β value	Standard Error	p-value
TAT complexes	-0.004	0.003	0.19
Fibrinogen	-0.002	0.003	0.57
TxB_2	-0.005	0.004	0.29

TAT: thrombin-antithrombin III, TxB_2: thromboxane B_2, β: regression coefficient.

Discussion

Our study examined the hemostatic efficacy of modified polyurethane foam by measuring different coagulation parameters in a human model. The coagulation parameters showed no differences between the PU foam and gelatin- or collagen-based materials. The differences between the parameters measured from the test materials and the baseline values however, were significant, indicating that all tested materials substantially increased hemostasis supplementary to the activation induced by the wound area.

A lot of research has been done on the hemostatic properties of biomaterials like polyurethane [5, 12, 13]. The polyurethane foam we tested was combined with 55 wt% PEG to increase the absorbing properties of the foam. The higher absorbability should increase the concentration of endogenous coagulation factors and platelets. The hemostatic effect of cellulose- and polysaccharide-based hemostatic agents is partially based on this mechanism [14, 15].

We used a human *in vivo* model in which it is possible to compare different hemostatic agents in similar wounds. The use of extraction sockets of upper and lower molars has the advantage that the test materials could be analysed under similar conditions within the same patient. We used coagulation parameters to determine hemostatic efficacy because time to hemostasis could not be accurately measured in this split mouth model. This could be considered a drawback as time to hemostasis is the most essential parameter.

The different structures of the test materials made it impossible to ensure complete blinding of the surgeons. We do not expect that this has influenced the results because the time in the wound was exactly 2 minutes for all test materials and the parameter values were measured by ELISA. Because the outcome of the ELISA was determined on numerical coded samples by computer measurement, the possible bias was restricted to a minimum for both surgeons and researchers.

We compared the PU foam with a collagen and gelatin hemostatic agent. These agents are together with oxidized regenerated cellulose traditionally the most widely used hemostatic agents in surgery [16]. They promote platelet aggregation and coagulation by providing a

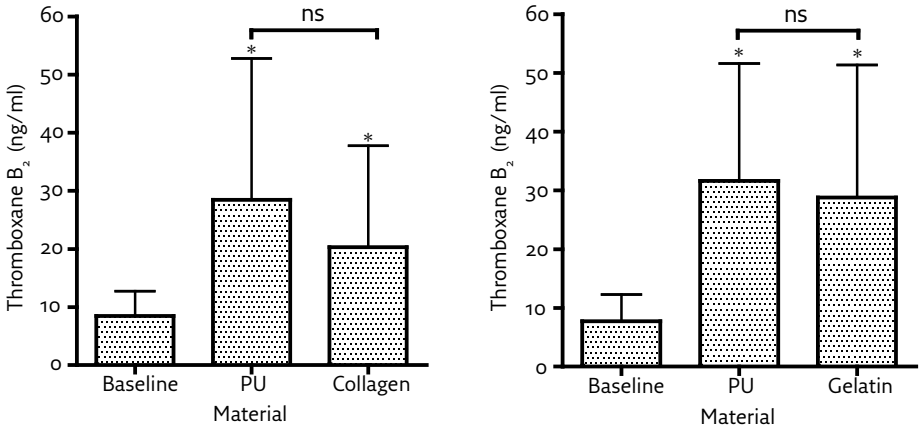

Figure 3. Mean (± SD) thromboxane B_2 concentrations for the different test materials and baseline samples. PU: polyurethane foam, ns: not significant. * = significant compared to baseline.

three-dimensional meshwork for clotting to take place [17]. The oxidized regenerated cellulose was not tested in this study because the structure of the material made it impossible to obtain plasma samples after centrifugation.

There is no consensus on which of these materials is the best hemostatic agent [18, 19], although some studies indicated that collagen is more effective than gelatin and oxidized regenerated cellulose [20, 21]. In this study no differences were seen in efficacy between the materials. The study design is unsuitable for comparison between collagen and gelatin but the differences appear to be minimal.

The mean baseline concentrations for the different coagulation parameters show great differences from the mean concentrations that are reported for circulating blood. The mean concentration of TAT complexes in circulating blood is 1.0 - 4.1 µg/L [22], whereas we found a mean baseline concentration of 0.55 µg/ml which is roughly 500 times higher. The mean baseline concentration of fibrinogen we found in this study is 0.51 mg/ml and therewith six times lower than the mean concentration in circulating blood of 3.0 mg/ml that is reported in the literature [23]. For TxB_2 the maximal estimate of the circulating concentration is 2.0 pg/ml [24]. The mean concentration of 8.1 ng/ml that we found in the baseline samples is 4000 times higher. These differences are due to activation of the coagulation cascade *in vivo* as the baseline blood was collected from a wound [9]. For this study these differences are of minor importance as every test was performed in a wound situation.

The extraction time could theoretically influence the baseline values as there is more time for the coagulation cascade to be completed. In our results we did not find a correlation between extraction time and baseline concentrations. This could be due to constant blood flow from the wound thus preventing a higher baseline value when the extraction time was longer. Another explanation would be that maximum baseline value was already reached after two minutes, which was the shortest extraction time.

The mouth is known as a fibrinolytic environment predominantly due to the saliva [25]. During our tests the influence of saliva was minimal due to continuous suction around the alveolus and furthermore equal for all tests. Therefore, this factor is not expected to have influenced the test results.
For this study only healthy subjects were included. The future use of the PU foam will be in patients with an impaired hemostasis. Future studies will have to show if the hemostatic efficacy of PU is strong enough to achieve complete hemostasis in those compromised patients. Further research should therefore also aim on improvement of the hemostatic efficacy of the PU. This could be done by combining the PU with a procoagulant substance.

Conclusions

In this study PU foam was compared with collagen and gelatin for its hemostatic efficacy in a human model. In this model, PU foam showed a similar hemostatic efficacy as the collagen and gelatin hemostatic agents. The synthetic PU is therefore a promising alternative for the nowadays used, animal derived, hemostatic agents.

Acknowledgements

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Chapter 4

Comparison of topical hemostatic agents in a rat tail tip model

Submitted

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Abstract

A broad variety of topical hemostatic agents are used in the surgical disciplines. We analysed the most widely used topical hemostatic agents and compared them to a newly developed, synthetic topical hemostatic agent based on polyurethane (PU). Furthermore, we tested PU enriched with the procoagulant substance chitosan to evaluate if this could increase its hemostatic efficacy.

The following topical hemostatic agents were compared using a rat tail tip model: Collagen, gelatin, oxidized regenerated cellulose, chitosan dressing, PU and PU with chitosan. The tail tip was fixated on a developed test device to ensure a constant and equal pressure of the test material on the wound. The mean bleeding time was determined and compared between the groups.

PU showed a mean bleeding time of 23.9 minutes. This was not significantly shorter or longer than gelatin (23.6 min), collagen (28.2 min) or oxidized regenerated cellulose (26.9 min). The addition of chitosan to PU did lead to the shortest mean bleeding time (21.5 min) but this was not significantly faster than PU without chitosan.

These results show that PU is a promising alternative for the most widely used topical hemostatic agents. Future studies will have to show if the addition of procoagulant substances like chitosan can significantly improve the hemostatic efficacy of PU.

Introduction

Many different topical hemostatic agents are used in the surgical disciplines. The most widely used agents are collagen, gelatin and oxidized regenerated cellulose (ORC). There is no consensus on which of these agents has the best hemostatic efficacy and each product has significant potential drawbacks. As a result, none of the products has become dominant over the other topical hemostatic agents [1].

A disadvantage of collagen and gelatin hemostatic agents is that they are animal derived and therefore carry the potential risk of pathogen transmission. The ORC can lead to inflammation of surrounding tissue and delay wound healing because of its low pH [2].

A newly developed fully synthetic topical hemostatic agent with a good biocompatibility could avoid these potential risks. Polyurethane (PU) is a synthetic, biodegradable material with good biocompatible properties [3]. The hemostatic capacity of this material was increased in an *in vitro* study by combining it with a high percentage of polyethylene glycol (PEG) [4]. Coagulation is initiated by platelets that adhere to the PU surface while the PEG enhances the absorbable properties of the PU which increases the concentration of the endogenous coagulation factors and platelets. The hemostatic effect of cellulose- and polysaccharide-based hemostatic agents is partially based on this mechanism [5,6]. Furthermore, PEG increases the hydrophilicity of the material and the greater volume of water within the polyurethane can allow for a greater amount of hydrolysis, and thus degradation, to take place [7,8]. The PU can be formulated into membranes and foams that can be directly applied to the wound surface.

In this study, the hemostatic efficacy of a PU foam was compared to a collagen, gelatin and ORC material in a standardized rat model. This was done to analyse the hemostatic efficacy of the new material compared to the most widely used hemostatic agents.

To analyse if this new hemostatic agent could be improved by adding a procoagulant substance, tests were also performed with PU combined with synthetic chitosan which is also known as Poly D-Glucosamine. Chitosan is the deacetylated form of chitin (poly-N-acetyl glucosamine). Both substances have a similar hemostatic action mechanism which is believed to result from mechanical sealing, vasoconstriction and the mobilization of erythrocytes, clotting factors and platelets to the site of the injury [9,10]. HemCon is a chitosan dressing (CD) and was also investigated to analyse if these results would match the PU with chitosan. HemCon is a relatively new hemostatic agent that has shown a good hemostatic efficacy in humans [11,12].

The materials were compared with each other using a previously described rat tail-tip model [13,14]. In this model the tail was transected at 5 mm from the tip and the test materials were applied to the wound until cessation of the bleeding occurred.

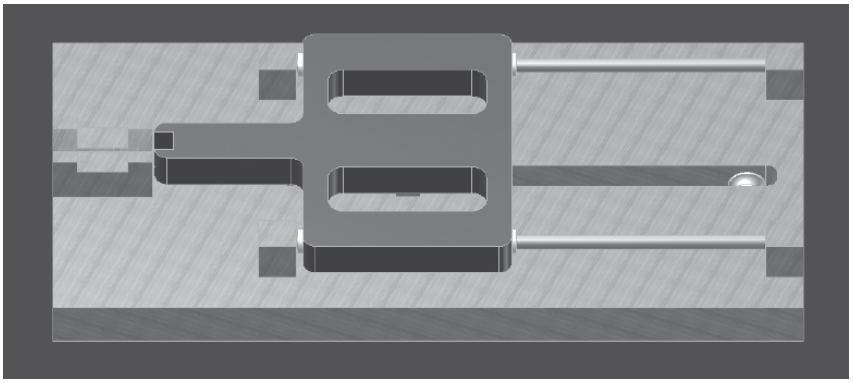

Figure 1. Schematic picture of the test device that was used to compare the different test materials. The tail was fixated on the platform on the left. The test material was placed in the sliding device in the middle. A weight that was connected to the sliding device via two pulleys ensured a constant pressure to the wound.

Materials and Methods

Animals

The study was approved by the Committee for Animal Experiments (University of Groningen, the Netherlands). Eighty male adult Wistar rats weighing 200 to 300 grams were obtained from Charles River, the Netherlands. The animals had unrestricted access to food and water.

Materials

Tests were performed with seven different test materials.

Hémocollagène (absorbable collagen hemostat; Septodont, Saint-Maur-des-Fossés, France) foams (1 cm^3) were used as collagen hemostatic agent.

Spongostan (absorbable gelatin foam; Johnson&Johnson, Skipton, United Kingdom) foams (1 cm^3) were used as gelatin hemostatic agent.

Surgicel (absorbable oxidized regenerated cellulose; Ethicon, Neuchâtel, Switzerland) knitted fabric (1.25 cm x 5 cm) was used as ORC hemostatic agent.

HemCon (chitosan-based wound dressing; HemCon Medical Technologies, Inc, Portland, Oregon, USA) was used as CD hemostatic agent.

Sterilux (sterile gauze compression bandages; Hartmann, Heidenheim, Germany) gauze pads (5 cm x 5 cm) were used as control agent.

The tested PU was a block-copolymer composed of urethane hard segments and co-poly-ether-ester soft segments. The soft segments (total molecular weight: 2.000 g/mol) consisted of 50% DL-lactide and 50% ε-caprolactone. PEG 1.000 was used as initiator for the soft segments synthesis, and after that PEG 20.000 was added in a mass ratio of 3 (first PU formulation) to 1 (PEG 20.000) to prepare a blend. The urethane segments were synthesized

with 1,4-butanediisocyanate (BDI) and 1,4-butanediol (BDO). They had a uniform length of 5 urethane moieties, which resulted in a PU with BDI-BDO-BDI-BDO-BDI urethane segments in the polymer. The PU was then dissolved in 1,4-dioxane. After dissolving, the solution was poured into a mold and cooled down to −18ºC. The solution was freeze-dried at 3 mbar to remove the 1,4-dioxane crystals, resulting in an highly porous foam with a porosity of 97% and an overall PEG-content of 55 wt%. Overall porosity was calculated after determining the weight and dimensions of the foams.
The PU foams with chitosan were made in the same way except for the addition of chitosan powder. After the PEG 20.000 was added, chitosan powder was added to the blend resulting in PU foams with a concentration of 50 mg chitosan per foam.
The PU foams had a cylindrical shape with a size of 1 cm^3 and were ultimately sterilized using ethylene oxide. The polymers and foams were manufactured by Polyganics BV (Groningen, the Netherlands).

Test device

A test device was developed for this test to ensure a constant and equal pressure of the test material on the wound (Figure 1). This test device consisted of a metal plate (30 cm x 15 cm x 2 cm) on which the tail of the rat and the test material could be fixated. The test material was placed on a sliding device on the metal plate. A weight of 155 g was attached to the sliding device via a pulley to ensure that a constant pressure would be applied to the wound. The weight created a constant pressure of 0.29 N/mm^2 that was applied to the wound. This was calculated out of the mass (0.155 kg) and acceleration of gravity (9.813 m/s^2), which gave a force of 1.521 N (0.155 * 9.813). This force was divided through the mean bleeding area of the wound (5.31 mm^2) which led to a pressure of 0.29 N/mm^2 ($2.9 * 10^5$ Pa). For this calculation we assumed that the friction in the device was negligible.

Study protocol

The eighty rats were randomly assigned to one of eight test groups of 10 animals each. From the eight test groups, seven were treated with a test material and one served as a negative control group and received no treatment. The tested materials were collagen, gelatin, oxidized regenerated cellulose, a chitosan dressing, gauze, PU foam and PU foam combined with chitosan.
The animals were anesthetized by inhalation of 5% isoflurane and weighed before surgery. During the surgery anesthesia was maintained by inhalation of 2-2.5% isoflurane. The animals were placed in a prone position on a platform with their tails resting 10 cm below the surface on the test device. A warming mat was placed under the rat to keep the temperature of the rat at 37º C. The rat tail was fixated on a platform on the developed test device with tape. The tail tip was placed 7 mm over the edge of the platform and transected at 5 mm from the tip. The mean diameter of the tail tip was 2.6 mm (SD: 0.24) and the mean weight of the transected tail-tip was 0.016 g (SD: 0.003).

Table 1. Mean bleeding time and standard deviation of the tested materials in order of efficacy.

Material	Mean bleeding time (min)	Standard deviation
PU + Chitosan	21,5	3,75
Gelatin	23,6	2,95
PU	23,9	5,49
ORC	26,9	4,20
CD	28,1	4,28
Collagen	28,2	7,63
Gauze	35,3	4,11
No material	54,5	4,86

PU, polyurethane; ORC, oxidized regenerated cellulose; CD, chitosan dressing

After transection of the tail, the test material was applied to the wound during three minutes. After three minutes the test material was removed and the wound was blotted with filter paper. This was repeated every minute until no blood was observed on the paper upon blotting. The time from the tail transection to the bleeding cessation was the bleeding time. During the experiment the tested material was replaced by a fresh one every ten minutes to prevent that the materials got fully saturated with blood and consequently might loose their hemostatic effect. After completion of the experiments, the animals were sacrificed by intravenous administration of 0.5 ml of pentobarbital sodium (200 mg/ml).

Statistical analysis

Statistical analysis was performed using SPSS 20.0. The mean bleeding time of PU was compared to the other materials with the Mann-Whitney-*U*-test. Using the Bonferroni correction a p value less than (0.05/5=) 0.01 was considered statistically significant. For the second comparison including the PU with chitosan a p value less than (0.05/3=) 0.017 was considered statistically significant.

The hemostatic agents collagen, gelatin, ORC and CD were compared using a Kruskal-Wallis test. A p value less than 0.05 was considered statistically significant for this test.

Results

The mean bleeding time and standard deviation was calculated per material and presented in table 1 in order of efficacy.

PU was first compared to collagen, gelatin, ORC, gauze and the control group without material. This showed that the bleeding time of PU was not significantly different from collagen (p=0.24), gelatin (p=0.70) and ORC (p=0.27). The difference with gauze and the control group without material was significant with a p value < 0.001 (Figure 2).

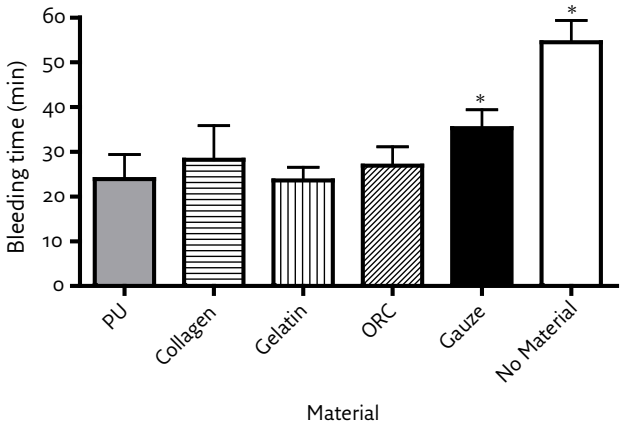

Figure 2. Schematic representation of the mean bleeding time and standard deviation for the different test materials. PU, polyurethane; ORC, oxidized regenerated cellulose. *p < 0.001 compared with PU.

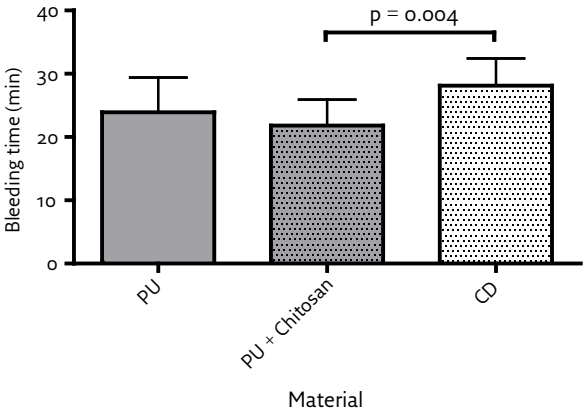

Figure 3. Schematic representation of the mean bleeding time and standard deviation for PU with and without chitosan and CD. PU, polyurethane; CD chitosan dressing.

PU was also compared to the PU that was combined with chitosan to analyze if this would improve the hemostatic efficacy. Furthermore, the chitosan dressing (CD) HemCon was included in this comparison to evaluate how this material would relate to the PU with chitosan (Figure 3). The bleeding times of PU with and without chitosan were not significant (p=0.27). The difference between PU and CD was also not significant (p=0.13). The PU with chitosan did have a significantly shorter bleeding time than CD with a p value of 0.004. The differences between collagen, gelatin, ORC and CD were not significant (p=0.14).

Discussion

This study was done to compare the hemostatic efficacy of the most widely used topical hemostatic agents with a newly developed synthetic hemostatic agent based on polyurethane. Tests were also performed with the polyurethane hemostatic agent combined with chitosan to analyse if the hemostatic efficacy could be improved by adding a procoagulant substance. We found that the mean bleeding time of PU was not significantly longer or shorter than the most widely used topical hemostatic agents. The addition of chitosan to PU did lead to a shorter mean bleeding time but this difference was however not significant compared to the standard PU foam. Thus, the addition of chitosan to the PU foam could not improve its hemostatic efficacy.

Previous studies on the hemostatic efficacy of widely used topical hemostatic agents showed various results. A number of studies concluded that the hemostatic agents had comparable results [15-17]. Several studies found collagen to be the most effective topical hemostatic agent [4,18,19]. Other studies pointed out chitin as the most effective topical hemostatic agent [20],[1,21].

Our study also showed no clear favourite amongst the tested topical hemostatic agents. The addition of chitosan to the PU foam could not improve its hemostatic efficacy. This could be caused by mixing the chitosan through the foam. As a result the chitosan concentration at the surface of the foam might not have been high enough to exert an additional effect. Future studies with a higher concentration of chitosan or a layer of chitosan at the surface will have to show if the addition of chitosan is a possibility to increase the hemostatic efficacy of PU.

The chitosan-based dressing HemCon showed a significantly longer bleeding time than the PU foam with chitosan. This might be explained by the hemostatic action mechanism of HemCon. The product becomes sticky upon contact with blood and creates an enhanced tissue adhesion [22]. In the used test model, the hemostatic agent had to be removed from the wound regularly to analyse if the wound was dry. The sticky property of the chitosan could have led to partial removal of the blood clot and thus a longer bleeding time.

Our bleeding model was modified from other studies [13,14]. The most important change was the use of a test device that was developed for this purpose. The test device ensured a constant pressure to the wound that was equal for all test materials. This is a major improvement because compression by the researcher inevitably leads to varying pressure to the wound. The removal of the tail at 5 mm from the tip led to a wound diameter with a low standard deviation and thus to comparable and reproducible wounds.

A drawback of this model was that the hemostatic agent had to be removed every minute to check if the wound was dry. This method implied that a part of the blood clot was removed every time the wound was checked. This effect was equal for all test materials but might have disadvantaged the chitosan which becomes sticky upon contact with blood [22].

Conclusions

The PU foam showed a comparable mean bleeding time as the widely used hemostatic agents in this model. The PU combined with chitosan showed the shortest mean bleeding time but this was not significantly different from PU. We conclude that PU is a promising alternative for the most widely used topical hemostatic agents. Future studies will have to show if the addition of procoagulant substances like chitosan can significantly improve the hemostatic efficacy of PU.

Acknowledgements

The authors gratefully acknowledge R. de Graaf, J. Jankie, and B.C. Bhat for the development and production of the PU foams. This study was financially supported by Polyganics BV and the University Medical Center Groningen, the Netherlands.

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Chapter 5

Hemostatic action of polyurethane foam with 55 wt% polyethylene glycol compared to collagen and gelatin

Submitted

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Abstract

For most topical hemostatic agents the mechanism of hemostatic action is not fully understood. This work aimed to investigate the hemostatic mechanism of action and viscoelastic properties of Polyurethane foam (PU) in comparison to the widely used collagen and gelatin. The hemostatic mechanism of action of the materials was tested using human whole blood and platelet-poor plasma (PPP). The ability of the hemostatic agent to exert pressure on the wound was quantified in terms of its viscoelastic properties both under dry and wet conditions using a low load compression tester (LLCT).

It has been shown that collagen and PU initiate hemostasis through both thrombocyte aggregation and contact activation of the coagulation cascade. Gelatin did not show improved thrombocyte aggregation or initiation of the coagulation cascade compared to the negative control group. PU is more firm under wet conditions and shows more springback than collagen and gelatin.

We conclude that PU is promising as a topical hemostatic agent because it initiates both the coagulation cascade and thrombocyte aggregation. Furthermore, it has favorable viscoelastic properties compared to collagen and gelatin which leads to increased pressure on a wound.

Introduction

Topical hemostatic agents are often used to control bleeding within the different surgical disciplines. The mechanism of hemostatic action however, is not fully understood for most topical hemostatic agents.

Under normal conditions, hemostasis is achieved by thrombocyte adherence to the subendothelium and activation of the coagulation cascade by Tissue Factor (TF). The initial clot is formed by the thrombocytes while TF reacts with factor VII to activate factor X which further activates the coagulation cascade. This leads to the formation of fibrin and stabilization of the clot [1]. The coagulation cascade can also be activated when blood comes into contact with a negatively charged surface. This contact activation system is traditionally known as the intrinsic pathway [2].

When the natural hemostatic process in a patient is insufficient, hemostatic agents can be used to accomplish hemostasis. The most often used topical hemostatic agents include collagen and gelatin. These hemostatic agents have different modes of action. For collagen, the mechanism of action is believed to be attraction and aggregation of thrombocytes. Hemostatic agents based on gelatin provide a physical matrix in which a clot can form while absorbing surrounding fluids [3].

We have been studying the hemostatic efficacy of Polyurethane foam (PU) with uniform hard segments composed of butanediol and 1,4-butanediisocyanate and soft segments of DL-lactide and ε-caprolactone combined with 55 wt% Polyethylene Glycol (PEG) [4,5]. In these studies we found that a high percentage of PEG increased the hemostatic capacity of PU and that the hemostatic efficacy of this material was comparable to collagen and gelatin hemostatic agents. We also noticed that PU kept its shape and firmness after contact with blood while the collagen and gelatin became soft and mushy and thus provides less pressure to the wound. Therefore, we developed a test set-up to objectify these notifications.

We hypothesize that the PU provides a surface to which thrombocytes can adhere and might initiate the coagulation cascade through contact activation. Furthermore, we expect that PU keeps its firmness and elasticity under wet conditions and thereby provides more pressure to the wound. The aim of the current study is to test our hypothesis and investigate the hemostatic action mechanism and viscoelastic properties of the PU foam.

We used three different tests in which we compared the PU to collagen and gelatin. The first test was performed with human whole blood to analyse the influence of the test materials on thrombocyte aggregation and activation. The second test was done with platelet-poor plasma (PPP) to analyse the initiation of the coagulation cascade in the absence of sufficient thrombocytes, by the different test materials. The third test was done with a low load compression tester (LLCT) to investigate the viscoelastic properties of the test materials both under dry and wet conditions. LLCT has been used in earlier studies to measure microbial biofilm properties and the viscoelastic properties of human lenses [6,7].

Materials

Tests were performed with PU, Hémocollagène (absorbable collagen hemostat; Septodont, Saint-Maur-des-Fossés, France) and Spongostan (absorbable haemostatic gelatin sponge; Ethicon, Somerville, NJ, USA).
The tested PU was a block-copolymer composed of urethane hard segments and co-poly-ether-ester soft segments. The soft segments (total molecular weight: 2.000 g/mol) consisted of 50% DL-lactide and 50% ε-caprolactone. PEG 1.000 was used as initiator for the soft segments synthesis, and after that PEG 20.000 was added in a mass ratio of 3 (first PU formulation) to 1 (PEG 20.000) to prepare a blend. The urethane segments were synthesized with 1,4-butanediisocyanate (BDI) and 1,4-butanediol (BDO). They had a uniform length of 5 urethane moieties, which resulted in a PU with BDI-BDO-BDI-BDO-BDI urethane segments in the polymer. The PU was then dissolved in 1,4-dioxane. After dissolving, the solution was poured into a mold and cooled down to −18ºC. The solution was freeze-dried at 3 mbar to remove the 1,4-dioxane crystals, resulting in an highly porous foam with a porosity of 97% and an overall PEG-content of 55 wt%. Overall porosity was calculated after determining the weight and dimensions of the foams. The foams had a cylindrical shape with a size of 1 cm^3 and were ultimately sterilized using ethylene oxide. The polymers and foams were manufactured by Polyganics BV (Groningen, the Netherlands).
All materials were processed to cylindrical disks with a diameter of 10 mm and a weight of 0,01 gram to ensure that the same amount of material was used for each test. For the PU this led to a thickness of 2.5 mm. The collagen samples had a thickness of 3.3 mm and the gelatin samples had a thickness of 5 mm. From each material, 70 samples were processed and tested.
For the analysis of the viscoelastic properties, the original dimensions of the materials were preserved. The collagen and gelatin samples had the shape of a cube which measured 1 cm^3. The PU had a cylindrical form with a diameter and height of 10 mm.

Methods

To analyse the hemostatic action mechanism and viscoelastic properties of the test materials, three different tests were done. For the first test, a thrombocyte count of human whole blood was performed after contact with the test materials. For the second test, time to hemostasis in thrombocyte-poor plasma was measured following activation with the test materials. For the third test, a low load compression test (LLCT) was used to analyse the viscoelastic properties in wet and dry conditions.

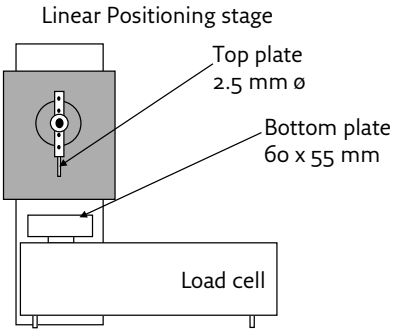

Figure 1. Frontal view of the LLCT, showing the main components of the system.

Thrombocyte count of human whole blood

Blood preparation

The study was approved by the ethics committee of the University Medical Center Groningen, the Netherlands. The blood that was used for this study was obtained from six healthy adult volunteers. Exclusion criteria were a known disease or use of drugs that could have influence on blood coagulation. Each volunteer donated 20 ml of blood which was tested for normal thrombocyte count. To prevent the blood from instant clotting, heparin was added to a concentration of 1.5 IU of heparin per ml blood. The blood was then divided over 40 eppendorf vials, 0.5 ml per vial and kept at room temperature. The tests were finished within two hours after the blood was obtained. At the start of each test 0.75 IU of protamine was added to the vial with blood to neutralize the effect of heparin.

Test device

An Amelung-coagulometer KC-4 (Lemgo, Germany) was used to rotate the tubes with 0.5 ml whole human blood at a constant temperature of 37 ºC. After the protamine was added, the test materials were placed in the tube with blood during 120 seconds. With the coagulometer four tests could be done simultaneously. In each test round a PU, collagen and gelatin sample were tested along with a control without material in a random order. After rotation for 120 seconds the materials were removed from the tube with blood. A thrombocyte count was then performed with the blood cell analyzer Medonic CA 530 (Medonic, Sweden) to determine the amount of free and thus unaggregated thrombocytes. In 14 of the 140 samples the thrombocyte count failed due to an insufficient amount of remaining blood or advanced hemostasis of the blood. These samples were excluded from the study.

Coagulation time of thrombocyte poor plasma

Preparation of thrombocyte poor plasma

Five units of citrated human plasma were obtained from the blood bank (Sanquin, Groningen, the Netherlands) and pooled. This thrombocyte poor plasma contained less than $30 * 10^9$/L

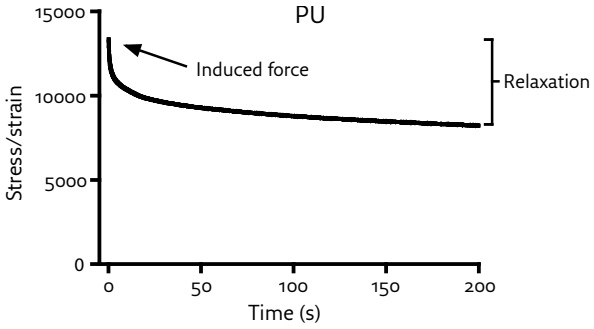

Figure 2. Example of a stress relaxation curve for PU out of which the percentage of stress relaxation is calculated. PU, polyurethane.

thrombocytes (Normal: 150-350 * 10^9/L). The thrombocyte poor plasma was divided over different plastic tubes, 450 µl per tube and kept at room temperature.

Test device

The tubes with thrombocyte poor plasma were placed in the Amelung-coagulometer KC-4 (Lemgo, Germany) at 37º C and incubated with one of the different test materials during 15 minutes. Hereafter, the test material was removed from the PPP and 10% v:v of $CaCl_2$ at a concentration of 300 mmol was added to neutralize the citrate. Tests were also performed with a negative control group without material and a positive control group with a glass tube. Due to the negative charge, glass is a strong activator of the contact activation system [8]. The coagulometer was then used to rotate the cups with PPP at a constant temperature of 37 ºC. A metal control ball was placed in the PPP sample. When coagulation of the sample led to a higher viscosity the ball was trapped by the fibrin. This was the endpoint of the test which was recorded by electromagnetic detection and led to the coagulation time. When the endpoint was not reached after 1200 seconds, the test was stopped and excluded from the study. This was the case in 10 of the 135 samples.

Analysis of viscoelastic properties

Test device

The viscoelastic properties of the test materials were analysed with a low load compression tester (LLCT). The LLCT consists of a linear positioning stage (Intellistage M-511.5IM; Physik instrumente, Karlsruhe, Germany) which is connected to a cylindrical moving upper plate with a diameter of 2.5 mm (Figure 1). A bottom stationary plate is fixed to an automatic force-compensating balance (SW 50/300, Wipotec, Kaiserslautern, Germany). The load cell and linear positioning stage were interfaced to a PC for data acquisition and control using LabVIEW 7.1. Movement of the top plate and force registered by the load cell were computer-stored for further analysis.

Table 1. Mean thrombocyte count of the test materials after contact with human whole blood.

Material	Number of tests	Thrombocytes (x 10^9/L)
No material	32	213.8 ± 28.8
PU	29	178.0 ± 32.4
Collagen	30	187.2 ± 25.3
Gelatin	35	200.7 ± 31.4

PU, polyurethane.

Stress-strain test

Tests were performed both under dry and wet conditions in a random order. The tests under wet conditions were done with materials that were submerged in demineralised water for 200 s. All materials were fully soaked with demineralised water after this time period. The mean percentage of demineralised water was 98% in the collagen foam, 90.5% in the gelatin foam, and 95.5% in the PU. The test material was placed on the bottom plate and positioned under the center of the top plate. The LLCT was then activated and the top plate moved downward with a constant speed of 10 µm/s. After the top plate made contact with the foam it started compressing it at 0.1%/s till a stress of 90 kPa was reached. The total deformation of the material was then recorded at 90 kPa.

Stress-relaxation test

Tests were performed under wet conditions in a random order. The test material was placed on the bottom plate and positioned under the center of the top plate. The LLCT was then activated and the top plate moved downward with a constant speed of 10 µm/s. When the top plate made contact with the foam, it imposed a deformation of 30% in 0.5 s. The loading force which is a parameter for the amount of springback was then monitored for 200 s (Figure 2). Out of the loading force the percentage of stress relaxation was calculated.

Statistical analysis

Statistical analysis was performed using SPSS 18.0. Group differences for all tests were calculated by the Mann-Whitney-U-test. P values less than 0.05 were considered statistically significant.

Results

Thrombocyte count human whole blood

To analyse the influence of the materials on thrombocyte aggregation and adhesion the amount of free thrombocytes in the blood samples was measured after a contact period of 120 seconds. A low amount of free thrombocytes indicates a high amount of aggregated or

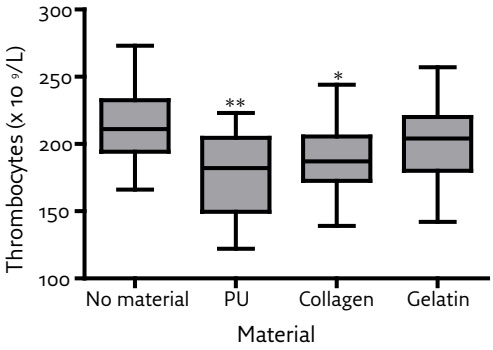

Figure 3. Schematic representation of the amount of thrombocytes for the test materials after contact with human whole blood. For each group, the line in the middle of the box represents the median. The lower and the upper edges of the box are 1st and 3rd quartile, respectively. The fences are drawn to the nearest value not exceeding 1.5 (interquartile range) * $p < 0.01$ compared with control group (No material). ** $p < 0.001$ compared with control group (No material). PU, polyurethane.

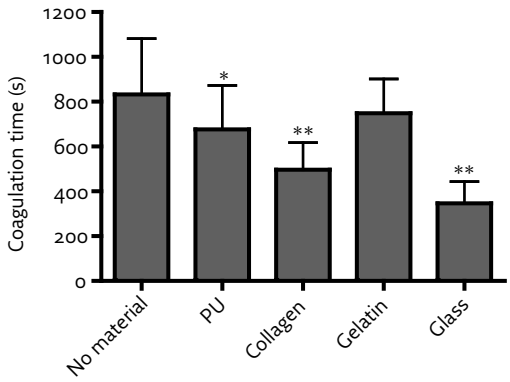

Figure 4. Mean coagulation time of the test materials in platelet-poor plasma. Mean standard deviations are plotted as error bars. * $p < 0.03$ compared with negative control group (No material). ** $p = 0.001$ compared with negative control group (No material). PU, polyurethane.

adhered thrombocytes. The mean amount of free thrombocytes was calculated and shown in table 1. The results are also presented in a boxplot (Figure 3).

The lowest mean amount of thrombocytes was found in the blood samples that had been in contact with PU. This was significantly lower than the mean amount of thrombocytes in the control group ($p < 0.001$). The mean amount of thrombocytes in the blood samples that had been in contact with collagen was also significantly lower than the mean amount in the control group ($p < 0.01$). Between gelatin and the control group the mean amount of thrombocytes was not significant ($p = 0.08$).
The difference in mean thrombocyte amount was not significant between PU and collagen ($p = 0.36$). The difference between PU and gelatin was significant ($p = 0.02$), whereas the difference between collagen and gelatin was not statistically significant ($p = 0.07$).

Coagulation time pooled plasma

The influence of the test materials on the coagulation cascade was analysed with platelet-poor pooled plasma. The coagulation time was measured for the different materials. Means and standard deviations are noted in table 2 and presented as bar chart in Figure 4.

Table 2. Mean coagulation time of the test materials in platelet-poor plasma.

Material	Number of tests	Coagulation time (s)
No material	14	832.6 ± 248.8
PU	33	676.1 ± 196.4
Collagen	33	496.2 ± 121.1
Gelatin	30	748.6 ± 153.1
Glass	15	346.5 ± 97.1

PU, polyurethane.

The mean time to coagulation was the highest for the negative control group in which no material was incubated. The mean coagulation time for collagen was significantly lower than the negative control group with a p-value < 0.001. The PU also showed a significantly faster coagulation than the negative control group ($p = 0.03$). The coagulation time of gelatin did not differ significantly from the negative control group ($p = 0.15$). The positive control group in which the plasma was incubated with glass showed a significantly faster coagulation than the negative control group and the other test materials ($p < 0.001$).
The coagulation time of collagen was significantly lower than PU and gelatin ($p < 0.001$). Between PU and gelatin the difference was not significant ($p = 0.13$).

Analysis of viscoelastic properties

Stress-strain test

The viscoelastic properties of the test materials were analyzed by performing a stress-strain test under dry and wet conditions. Mean deformation under 90 kPa compressive stress from 12 measurements is presented in a bar chart in figure 5.
The PU foam showed the highest mean deformation under dry conditions. The deformation of PU increased when the test was performed under wet conditions ($p < 0.001$). Collagen and gelatin foams showed significantly lower deformation as compared to PU foam in dry conditions ($p < 0.001$). The deformation under wet conditions for collagen and gelatin foams increased 4 and 9 times respectively ($p < 0.001$).

Stress-relaxation test

The springback capability of the materials was analyzed under wet conditions after rapid deformation of 30%. The springback was recorded in terms of percentage stress relaxation and presented in a boxplot in figure 6. The lower the relaxation, the higher is the springback capability. The highest percentage of stress relaxation was recorded for the tests that were done with gelatin. Collagen gave less relaxation and the lowest percentage of relaxation was seen for PU. The differences between the materials were statistically significant ($p < 0.001$).

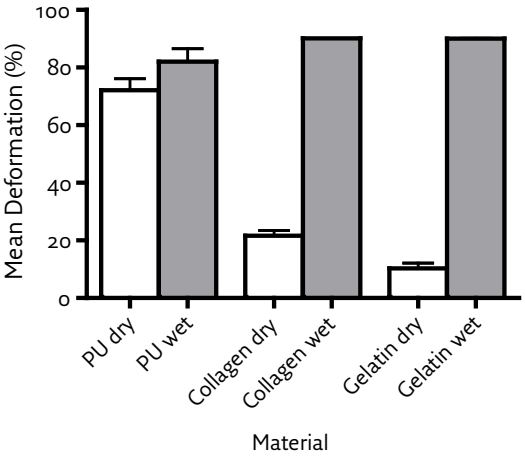

Figure 5. Mean deformation of both dry and wet PU, collagen and gelatin foams under a compressive stress of 90 kPa. Standard deviations of 12 measurements are plotted as error bars, bars with the maximum allowed deformation of 90% do not possess any standard deviation. PU, polyurethane.

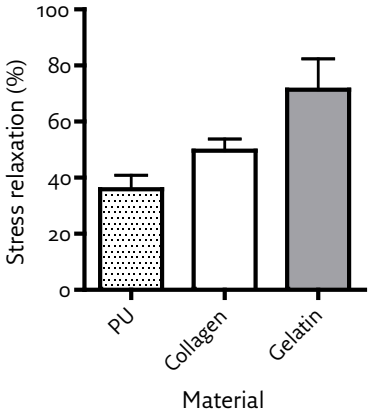

Figure 6. Mean percentage stress relaxation in 200 seconds under wet conditions for different types of foam at 30% deformation. Mean standard deviations of 15 measurements are plotted as error bars. PU, polyurethane.

Discussion

In this study, PU showed a significant increase in thrombocyte aggregation and activation of the coagulation cascade compared to the negative control group. Collagen showed similar results but led to a significantly faster coagulation in the pooled plasma test. This indicates that PU and collagen are thrombogenic materials due to their surface reaction with blood components. Gelatin did not show any significant differences in thrombocyte aggregation and initiation of the coagulation cascade compared to the negative control group. The analysis of the viscoelastic properties showed that the deformation of PU slightly increases under wet conditions while the deformation of collagen and gelatin was increased multiple times under wet conditions. The stress relaxation was the lowest for PU, followed by collagen and gelatin. This indicates that PU has the highest amount of springback after impression under wet conditions.

In the *in vivo* situation, vessel injury leads to the rapid binding of thrombocytes to the subendothelium [1]. The coagulation cascade is initiated by TF which activates FX after reaction with FVII. This leads to fibrin deposition and stabilization of the clot [1]. The initial clot is thus formed by thrombocytes and stabilisation of the clot occurs via the coagulation cascade. A hemostatic agent should therefore ideally promote both the aggregation of thrombocytes and initiation of the coagulation cascade.

In this study both PU and collagen show these characteristics. Collagen is already known as an effective hemostatic agent although its mechanism of action is predominantly attributed to the promotion of thrombocyte aggregation [3]. This study showed that the hemostatic efficacy of collagen also arises out of initiation of the coagulation cascade. An early study by Wilner et al. already showed that factor XII can be activated by collagen in thrombocyte poor plasma and lead to coagulation via the intrinsic pathway of the coagulation cascade [9]. Although some studies [10-12] questioned the role of factor XII in *in vivo* clot formation, a study by Renné et al. pointed out that the FXII-induced intrinsic coagulation pathway is important for clotting *in vivo* [13]. Other studies also found that factor XII contributes to thrombus formation and activation of the coagulation cascade [14,15].

The fact that PU also leads to increased thrombocyte aggregation and initiation of the coagulation cascade is a confirmation of our hypothesis that the PU provides a surface to which thrombocytes can adhere and initiates the coagulation cascade through contact activation.

The results of gelatin fell short compared to collagen and PU in this study. The gelatin showed less aggregation of thrombocytes in the human whole blood and longer coagulation time with platelet-poor plasma compared to collagen and PU.

A previous *in vitro* study also showed very low platelet deposition for gelatin when the material was exposed to platelet rich plasma [16]. Other studies state that the hemostatic action mechanism of gelatin is probably mostly mechanical and seems likely to involve physical surface effects rather than any action on the blood clotting mechanism [3,17].

Compression of a wound is known to be effective and important for hemostasis [18]. Therefore, it is important that a topical hemostatic agent keeps its firmness and shape after it is soaked with blood. The results in this study show that the mechanical stability of collagen and gelatin foam significantly decrease after getting soaked, whereas PU foam maintained its stability. Under dry conditions the PU foam was highly malleable and deformed the most easily, whereas collagen and gelatin would require higher forces. Under wet conditions both collagen and gelatin foam deformed up to the maximum allowed of 90% and would have deformed to much higher values in absence of this limit, indicating that the deformation under 90 kPa for PU foam is significantly lower as compared to collagen and gelatin foam.

In the stress relaxation test the materials were impressed for 30% because surgeons often manipulate the hemostatic agent before application to a wound. The 30% was chosen as an estimate of the extent to which surgeons generally manipulate the hemostatic agents. The tests were performed under wet conditions to mimic the *in vivo* situation in which the materials get soaked with blood after application to the wound.

The stress relaxation test showed that PU has the lowest stress relaxation, indicating that it has the highest amount of springback and therefore will exert more pressure on a wound even after getting soaked with blood. Collagen and gelatin foam on the other hand become mushy and formless under wet conditions and therefore might be much less successful in restricting the bleeding. We did not find any indications for possible compressive complications due to extreme swelling of gelatin and collagen which is often mentioned in the literature [3,19-21]. Furthermore, the stability of PU foam under wet conditions gives it better handling capacities than the mushy and formless collagen and gelatin foam.

The poor viscoelastic properties of gelatin are probably due to the production process in which denaturation of collagen leads to breaking of the triple-helix structure [22]. An *in vitro* study by Grover et al. also found that collagen has better viscoelastic properties than gelatin which is supported by our stress relaxation results [23]. PU is a synthetic material with attractive viscoelastic properties that can be controlled and modified during the production process [24]. We found in our study that the viscoelastic properties of PU were only very slightly altered under wet conditions. This corresponds to another *in vitro* study that showed that stress and elongation properties changed little when PU containing PEG was hydrated [25].

For the tests on hemostatic action mechanism the materials all had a weight of 0.1 gram. We used weight in stead of volume because weight could be more accurately measured. The materials had a different density which led to a larger volume of gelatin than the volumes of collagen and PU. This could be advantageous for gelatin because the total surface to which platelets can adhere was greater than the surfaces of collagen and PU. As gelatin was the weakest in both tests this variation has not altered the outcome of this study.

The analysis of thrombocyte activation was done with human whole blood. The unpredictable behaviour of blood *in vitro* led to early coagulation in some of the samples. Furthermore, in a few samples the test material soaked up the majority of blood leaving an insufficient amount of blood to accurately perform the thrombocyte count.

Initiation of the coagulation cascade was analysed with thrombocyte poor plasma to minimize the influence of the thrombocyte component on hemostasis. The fact that the PPP was not entirely free of thrombocytes could have influenced the test results. In some of the samples no coagulation occurred after 1200 seconds. We choose to end the test after 1200 seconds because no coagulation can be expected after this timeframe.

With the LLCT we were able to accurately analyze and compare the viscoelastic properties of the different test materials. The tests under wet conditions were performed with test materials that were soaked with demineralised water instead of blood for practical reasons. This could have had a minor effect on the test results but is unlikely to influence differences between foams.

This study showed that the good hemostatic efficacy of PU shown in previous studies is predominantly due to its effect on thrombocyte aggregation. The viscoelastic properties of the PU ensure good handling capacities and pressure to a wound. Future improvement of this material should therefore aim on its capacity to initiate hemostasis through contact activation.

Conclusions

This study showed that collagen and PU initiate hemostasis through both thrombocyte aggregation and contact activation of the coagulation cascade. Gelatin did not show improved thrombocyte aggregation or initiation of the coagulation cascade compared to the negative control group. PU is more firm under wet conditions and shows more springback than collagen and gelatin. We conclude that PU is promising as a topical hemostatic agent because it initiates both the coagulation cascade and thrombocyte aggregation and furthermore has favorable viscoelastic properties. Future improvement of PU should aim on its capacity to initiate hemostasis through contact activation.

Acknowledgements

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Chapter 6

In vivo degradation of polyurethane foam with 55 wt% polyethylene glycol

Submitted

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Abstract

Most topical hemostatic agents that are used during or after surgery are based on animal-derived products like collagen and gelatin. They carry the potential risk of pathogen transmission while adjustments in the production process of these materials are limited. A synthetic hemostatic agent based on polyurethane (PU) and polyethylene glycol (PEG) was developed to overcome these disadvantages. The goal of this study was to compare the degradation process of this biomaterial to collagen and gelatin hemostatic agents. Renal functions of the animals were determined to analyse the possible nefrotoxicity of PEG.

Samples of the test materials were implanted subcutaneously in both rats and rabbits. The animals were sacrificed at certain time intervals up to three years. At these time intervals the samples were located and explanted from the animals. Blood was taken from the animals for analysis of any abnormalities in urea and creatinine values. After fixation, the samples were histologically processed for microscopical evaluation.

The histological examination showed a comparable pattern of degradation for the different test materials. However, the degradation process was completed remarkably faster for collagen and gelatin than for PU. Remnants of gelatin and collagen were not seen in the samples after 12 weeks and 26 weeks, respectively. For PU, it took up to three years before microparticles of the material were no longer detected. All biomaterials showed a good biocompatibility and no severe foreign body reactions occurred. Mean urea and creatinine values stayed within the normal range during the three year investigation period.

The good biocompatibility and predictable pattern of resorption indicate that PU can be used as a topical hemostatic agent. However, a degradation time comparable to collagen and gelatin would be favorable.

Introduction

In all surgical disciplines, topical hemostatic agents are used to control local problematic bleeding. Most of these hemostatic agents are based on animal-derived products like collagen and gelatin. These materials carry the potential risk of pathogen transmission which can result in diseases like Creutzfeld-Jacob or bovine spongiform encephalopathy [1]. A hemostatic agent made of a synthetic yet biologically friendly material like polyurethane (PU) could prevent this risk. Furthermore, the possibility to vary the composition of synthetic biomaterials, allows for greater control over material properties and tissue responses.

Biodegradable PU with a total of 55 wt% polyethylene glycol (PEG) has been analysed for its hemostatic and mechanical properties in previous studies. The hemostatic efficacy of this material has shown to be comparable to topical hemostatic agents like collagen and gelatin [2,3]. The viscoelastic properties of PU were favorable compared to collagen and gelatin in an *in vitro* study as PU showed the highest springback capability under wet conditions [4]. Besides hemostatic and mechanical properties, characteristics like the bioresorption potential, the antigenicity and the tissue reactivity are important for a well balanced, effective hemostatic agent [5].

The goal of this study was to analyse the degradation process and the tissue response of PU with 55 wt% PEG and compare this to the degradation process and the tissue response of collagen and gelatin hemostatic agents.

The PU is composed of hard segments of 1,4-butanediol and 1,4-butanediisocyanate and soft segments of DL-lactide, ε-caprolactone and polyethylene glycol. The good biocompatibility of a comparable PU without PEG has been demonstrated in a previous *in vivo* study [6]. In this study the PU samples were resorbed almost completely after three years. A more rapid degradation period would be favorable to prevent the risk of secondary inflammation.

The degradation of PU can be accelerated by combining it with polyethylene oxide (PEO) [7]. This is attributed to the hydrophilic nature of PEO, because the presence of a greater volume of water within the polyurethane can allow for a greater amount of hydrolysis, and thus degradation, to take place [8,9]. PEO is also known as polyethylene glycol (PEG), depending on its molecular weight. In a pilot study in rabbits, a more rapid degradation was observed for PU with 23 wt% PEG compared to PU without PEG [10]. In a similar study, PU with 5 wt% PEG was tested which resulted in detectable foam remnants after one year [11]. We combined 55 wt% PEG with PU to further increase the hydrophilicity, and we expect that this material will show a more rapid bioresorption than the PU foams with lower percentages of PEG.

Topical hemostatic agents like collagen and gelatin usually degrade over a time period of 6 to 12 weeks [12]. The purpose of this study was to analyse the length of degradation of this PU foam and compare it to collagen and gelatin hemostatic agents, to determine whether the PU foam is suitable for controlling local bleeding during and after surgery. Furthermore, the antigenicity and tissue reactivity were evaluated. Renal functions of the animals were also examined at certain time intervals, because high amounts of PEG have been reported to induce nephrotoxicity [13].

Materials

Implantations were performed with PU, Hémocollagène (absorbable collagen hemostat; Septodont, Saint-Maur-des-Fossés, France) and Spongostan (absorbable haemostatic gelatin sponge; Ethicon, Somerville, NJ, USA).
The PU was a block-copolymer composed of urethane hard segments and co-polyether-ester soft segments. The soft segments (total molecular weight: 2.000 g/mol) consisted of 50% DL-lactide and 50% ε-caprolactone. PEG 1.000 was used as initiator for the soft segments synthesis, and after that PEG 20.000 was added in a mass ratio of 3 (first PU formulation) to 1 (PEG 20.000) to prepare a blend. The urethane segments were synthesized with 1,4-butanediisocyanate (BDI) and 1,4-butanediol (BDO). They had a uniform length of 5 urethane moieties, which resulted in a PU with BDI-BDO-BDI-BDO-BDI urethane segments in the polymer. The PU was then dissolved in 1,4-dioxane. After dissolving, the solution was poured into a mold and cooled down to −18ºC. The solution was freeze-dried at 3 mbar to remove the 1,4-dioxane crystals, resulting in an highly porous foam with a porosity of 97% and an overall PEG-content of 55 wt%. Overall porosity was calculated after determining the weight and dimensions of the foams. The foams used for implantation had a cylindrical shape with a diameter of 10 mm, a thickness of 1 mm and an average weight of 5 mg. The polymers and foams were manufactured and supplied by Polyganics BV (Groningen, the Netherlands), and were sterilized using the ethylene oxide process.
The collagen and gelatin foams were also processed to cylindrical discs with a diameter of 10 mm and an average weight of 5 mg to ensure that the same amount of material was inserted. The collagen samples had a thickness of 1,5 mm while the gelatin samples had a thickness of 2,5 mm. For traceability, a nondegradable polyamide suture (Ethilon®, 4-0, Ethicon) was attached to the discs as a reference.

Animals

Six-week-old male Wistar rats (n = 36) were used at time intervals up to 1.5 years (Table 1). In each rat six discs were implanted, in subcutaneous pockets, parallel to the spine in two rows of three. Half of the rats (n = 18) received six PU discs and the other half (n = 18) received two discs of each material (PU, collagen, gelatin). The position of the implants was rotated to exclude influences of the site of implantation. At each time interval four rats were terminated, two rats with only PU and two rats with PU, collagen and gelatin. We used eight time intervals up to 1.5 years for which we needed 32 rats. Four extra rats were implanted that served as back-ups in case of adversities. At 61 weeks one of the rats was terminated because of severe weight loss. The implants from this rat were not histologically analyzed. As no other adversities occurred, the three remaining back-up rats were terminated at the latest time interval for the rats at 1.5 years.
New Zealand White rabbits (n = 15) were used for the extended time intervals, because of their longer life span (Table 1). The animals were three months of age at the time of implanta-

tion. In each rabbit eight discs were implanted subcutaneously in two rows of four. Five rabbits received eight PU discs while the other ten rabbits were implanted with a combination of four PU discs, two collagen discs, and two gelatin discs. Similar to the rats, the position of the implants was also rotated in the rabbits. At the time intervals of 1 and 1.5 year, 2 rabbits were terminated and 4 and 7 rats, respectively. These two overlap time intervals were chosen to compare the tissue response and degradation of the materials between rats and rabbits.
In accordance to the rat series, two of the fifteen rabbits were implanted and served as backups in case of adversities. As no adversities occurred, the choice was made to terminate an extra rabbit at the 2.5 and 3 year time interval.
The rats and rabbits were obtained from Harlan Laboratories BV, the Netherlands. All animals were housed according to the Dutch national code of practice for animal welfare and had unlimited access to standard food and water. The experiment was approved by the Committee for Animal Experiments, University of Groningen, the Netherlands.

Methods

Subcutaneous implantation

All animals were weighed before implantation. The rats were anaesthesized with 2% isoflurane. Anesthesia for the rabbits was induced by intravenous administration of 1.0 mL pentobarbital (6%) and maintained with 2–3% isoflurane. The dorsal skin of the animals was shaved as to implement subcutaneous pockets parallel to the spine. The discs were placed in these pockets and closed with a suture. All wounds were inspected daily during the first week postoperatively. At the designated time intervals the animals were again weighed and anesthesia was induced. Venous blood was collected for analysis of the renal functions which was determined by measuring the blood urea nitrogen (BUN) and the creatinine concentration with a portable clinical analyzer (i-STAT 1 Analyzer; Abbott Laboratories, Abbott Park, IL, USA). The kidneys were then removed from the animal which caused death by bleeding. Kidney samples were taken and stored in either 4% phosphate buffered paraformaldehyde (PFA) or liquid nitrogen to be available for possible future analysis.
The explantation procedure for the samples was the same for rats and rabbits. The dorsal skin was shaved and cut open along the length of the spine. Implant sites were located using the blue polypropylene suture that served as implant reference. After macroscopical inspection the implants or the remains of, were explanted, including the overlaying skin. In some cases the suture could not be found and no indication of the biomaterial was seen. Therefore, the number of evaluated samples per time interval was not always equal. In table 2 and 3 the number of samples that could be evaluated per time interval is noted. The samples were fixated in 4% phosphate buffered PFA and subsequently embedded in GMA. Tissue blocks were cut perpendicular to the implant with a thickness of 2 µm and the sections were stained with Toluidin Blue or Toluidin Blue/Basic Fuchsin. Care was taken to orient the blocks precisely in order to determine the breakdown of the implants accurately.

Light microscopic evaluation of the samples was done using a semiquantitative scoring method (Table 2 and 3). All samples were observed and discussed by two investigators.

Statistical analysis

Statistical analysis was performed using SPSS 20.0. The Kruskal-Wallis test was used to compare the number of cell layers in the fibrous capsule between the materials. A p value less than 0.05 was considered statistically significant.

Results

Post-operative inspection of the animals revealed no inflammation around the implantation sites or impaired healing of the wounds at any time interval. The results of the histological examination were scored in a similar way as a previous study on the degradation of PU without PEG to facilitate comparison between the studies [6]. Table 2 and 3 present an overview of the histological examination and the rate of degradation is presented in Figure 1.

The histological examination showed a comparable pattern of degradation for the different test materials. The pattern of degradation could be divided in three different stages. These stages are encapsulation/infiltration, degradation/fragmentation, and resorption. The time to complete resorption was less for collagen and gelatin than for PU. The collagen and gelatin implants showed a similar degradation time until 12 weeks. From 26 weeks on, the degradation of gelatin progressed more rapid than the degradation of collagen.

The *encapsulation/infiltration stage* was seen in the collagen and gelatin implants during the first 2 weeks and in the PU implants as long as 12 weeks (Figure 2). The characterization of encapsulation of the biomaterial at this stage predominantly consisted of various numbers of fibroblast layers. These fibroblast layers would maintain their infiltration and encapsulation capabilities to smaller fragments of biomaterials as degradation sets in. Phagocytic activity by macrophages was seen in all biomaterials after 1 week. However, the concentration of macrophages was higher in the collagen and gelatin than in the PU foam. After 4 weeks the number of macrophages in the PU implants increased. These macrophages showed an increase in phagocytic activity after 12 weeks. A small number of acute inflammatory cells and foreign body giant cells (FBGCs) were seen around the biomaterials at the time intervals from 4 to 26 weeks. The fibroblastic cell layers around the biomaterials reached an average thickness of 4-6 cell layers. In table 2 the amount of cell layers at each time interval is presented for all biomaterials. The fibrous capsule was present around the collagen and gelatin materials during the first 2 weeks and around the PU implants up to 12 weeks. The difference in the amount of cell layers during week 1 ($p=0.91$) and week 2 ($p=0.83$) was not significant for the different biomaterials.

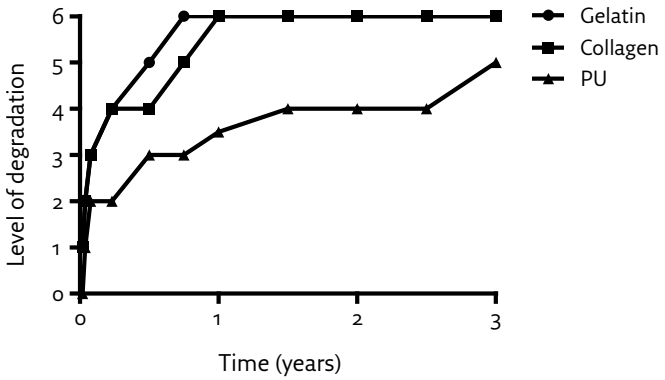

Figure 1. Overview of the level of degradation for the three biomaterials over time. 0: signs of degradation at the border of the material. 1: degradation in ½ of the material. 2: degradation in the centre of the material. 3: with partly loss of porous structure. 4: intracellular remnants of material. 5: signs of cellular activity indicative of degradation without visible material. 6: no signs of cellular activity indicative of degradation. PU, polyurethane foam.

Table 1. Overview of the terminated animals per time interval and their mean weight, BUN and creatinine at the time of sacrifice. BUN, blood urea nitrogen; SD, standard deviation.

Time interval	Mean weight with SD (grams)	Mean BUN with SD (mmol/L)	Mean creatinine with SD (µmol/L)
1 week (4 rats)	284.8 (± 6.8)	6.9 (± 0.42)	25.8 (± 4.9)
2 weeks (4 rats)	328.3 (± 25.7)	6.3 (± 1.09)	23.3 (± 4.1)
4 weeks (4 rats)	356.3 (± 34.5)	7.1 (± 0.39)	22.3 (± 3.8)
12 weeks (4 rats)	446.3 (± 43.2)	6.4 (± 0.26)	23.5 (± 3.4)
26 weeks (4 rats)	558.0 (± 45.6)	5.6 (± 0.32)	28.0 (± 4.2)
39 weeks (4 rats)	591.5 (± 51.5)	6.7 (± 0.60)	23.0 (± 4.2)
1 year (4 rats)	658.0 (± 34.2)	7.9 (± 0.50)	32.5 (± 7.0)
1 year (2 rabbits)	2900 (± 0)	5.1 (± 0.28)	101.5 (± 9.2)
1.5 years (7 rats)	584.9 (± 43.8)	9.3 (± 0.50)	41.9 (± 10.6)
1.5 years (2 rabbits)	3150 (± 353)	7.9 (± 1.1)	101.0 (± 7.1)
2 years (3 rabbits)	3333 (± 416)	7.2 (± 1.3)	92.3 (± 5.5)
2.5 years (4 rabbits)	3250 (± 191)	8.1 (± 0.5)	113.5 (± 22.8)
3 years (4 rabbits)	3600 (± 337)	5.9 (± 0.6)	107.0 (± 18.1)

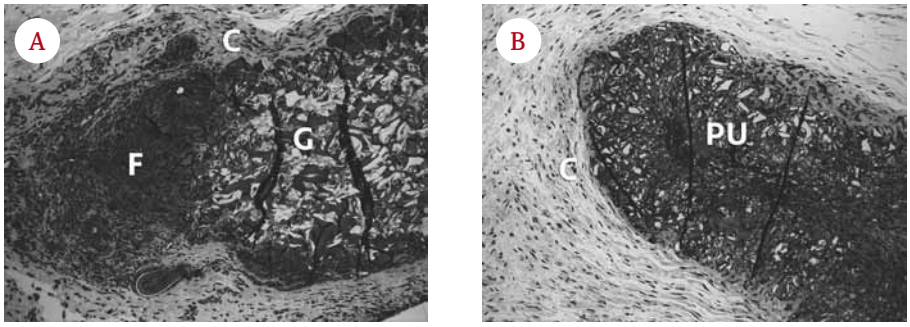

Figure 2. LM micrograph (10x) of a gelatin (G) sample and a PU sample after 1 week. A: Around the gelatin sample a capsule (C) is formed and the ingrowth of fibrous tissue (F) has started. B: A capsule (C) has also formed around the PU sample after 1 week but ingrowth is less clearly compared to gelatin. Also note the high density of PU compared to gelatin. PU, polyurethane foam.

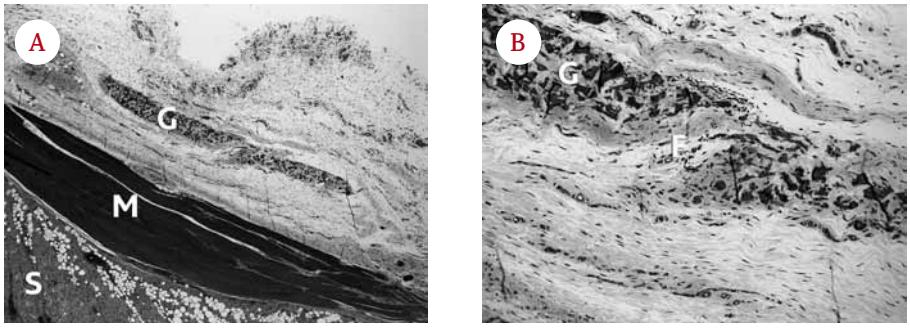

Figure 3. Overview of a gelatin (G) sample after 4 weeks. Figure 3A shows an overview (2.5x) of the gelatin sample after 4 weeks. Note the complete ingrowth of fibrous tissue (F) in the gelatin sample. Figure 3B (10x) shows a detail of the infiltrated gelatin sample. The disintegration of the biomaterial marks the start of the degradation/fragmentation stage. M, muscle. S, subcutaneous tissue.

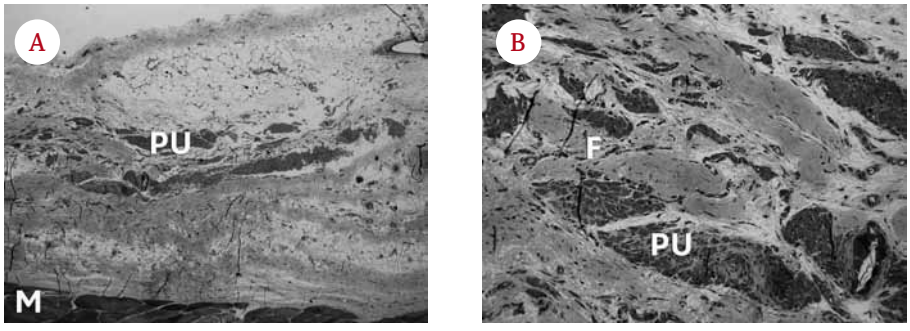

Figure 4. Overview of a PU sample after 39 weeks (A: 2.5x and B: 10x). Note the degradation and fragmentation of the biomaterial which is similar to the degradation stage of gelatin after 4 weeks. Ingrowth of fibrous tissue (F) can be seen in the complete area of the biomaterial. PU, polyurethane

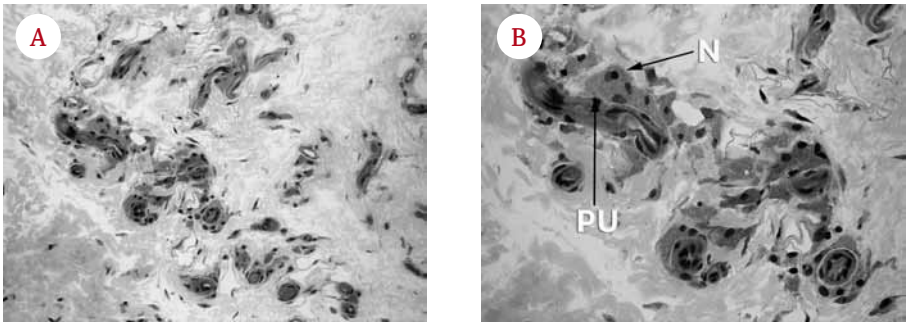

Figure 5. Detail (A: 20x and B: 40x) of the intracellular appearance of PU after 1.5 years in a rat. Note the nucleoli (N) of the macrophages that contain remnants of the biomaterial. PU, polyurethane foam.

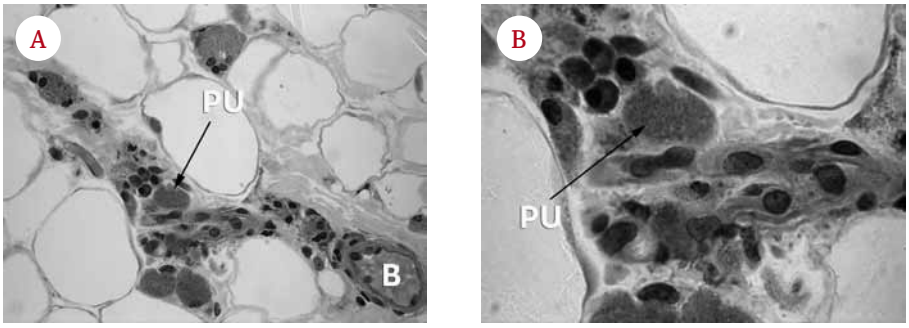

Figure 6. The intracellular configuration of PU after 2 years in a rabbit. Figure 6A (40x) shows the arrangement of macrophages containing PU near a blood vessel (B). Further magnification (100x) in figure 6B shows the granular aspect of the phagocytised biomaterial. PU, polyurethane foam.

The *degradation/fragmentation stage* began in the collagen and gelatin implants after 4 weeks while this commenced after 26 weeks with regards to the PU foam. The initial fibrous encapsulation was followed by a continuous ingrowth of fibrous tissue as a result of the continuous degradation and fragmentation process of the biomaterial. Early in this process vascularisation was observed, often near new fibrous tissue and in the vicinity of advanced degraded biomaterial. Major ingrowth of fibrous tissue in gelatin, seen at 4 weeks (Figure 3) corresponds to the ingrowth in PU foam after 26 to 39 weeks (Figure 4).
During this stage the macrophages showed the most phagocytic activity. As the resorption reached its final stage, the number of macrophages and their activity would also gradually decrease.

The *resorption stage* started after 12 weeks for the collagen and gelatin implants while resorption of the PU implants was seen after one year. The majority of fragmented particles of collagen and gelatin were phagocytosed by the macrophages at the 12 week interval. After 26

Table 2. Histological evaluation of the rat samples. For explanation, see legend on page 86.

Time interval	1 week			2 weeks			4 weeks			12 weeks			26 weeks			39 weeks			1 year			1.5 years		
Sample	**PU**	**Col**	**Gel**	**PU**	**Col**	**Gel**	**PU**	**Col**	**Gel**	**PU**	**Col**	**Gel**	**PU**	**Col**	**Gel**	**PU**	**Col**	**Gel**	**PU**	**Col**	**Gel**	**PU**	**Col**	**Gel**
Number of evaluated samples	15/16	4/4	4/4	14/16	4/4	3/4	14/16	4/4	3/4	13/16	4/4	4/4	14/16	4/4	4/4	13/16	4/4	4/4	16/16	4/4	4/4	30/30	6/6	6/6
Fibrous capsule	6.1 ± 0.9	5.6 ± 1.4	5.3 ± 1.2	5.8 ± 0.7	5.3 ± 1.7	4.9 ± 1.0	5.4 ± 0.6	-	-	4.5 ± 1.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tissue ingrowth	1	2	2	2	2	3	3	3-4	4	3-4	4	-	4	-	-	4	-	-	4/-	-	-	-	-	-
Macro-phages	1	2	2	1	1	1	2	2	2	2	2	1	2	2	2	3	1	0	2	1	1	2	0	0
FBGCs	0	0	0	0	0	0	1	1	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0
Cells WFA	1	2	2	1	2	1	1	3	2	2	2	1	2	1	1	2	1	0	1	0	0	1	0	0
LM degradation	0	1	1	1	2	2	2	3	3	2	4	4	3	4	5	3	5	6	3	6	6	4	6	6
Stage of degradation	E	E/I	E/I	E/I	E/I	E/I	E/I	D/F	D/F	E/I	R	R	D/F	R	R	D/F	R	R	F/R	R	R	R	R	R

Table 3. Histological evaluation of the rabbit samples. For explanation, see legend on page 86.

Time interval	**1 year**			**1.5 years**			**2 years**			**2.5 years**			**3 years**		
Sample	**PU**	**Col**	**Gel**	**PU**	**Col**	**Gel**	**PU**	**Col**	**Gel**	**PU**	**Col**	**Gel**	**PU**	**Col**	**Gel**
Number of evaluated samples	12/12	2/2	2/2	4/8	4/4	4/4	16/16	4/4	3/4	18/20	6/6	6/6	24/24	4/4	4/4
Fibrous capsule	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tissue ingrowth	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Macro-phages	1	0	0	1	1	1	1	1	1	1	0-1	0	0-1	0	0
FBGCs	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cells WFA	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0
LM degradation	4	6	6	4	6	6	4	6	6	4	6	6	5	6	6
Stage of degradation	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

Legend table 2 and 3

Fibrous capsule

Number of cell layers

Tissue ingrowth

0. No ingrowth
1. Ingrowth in ¼ of the material
2. Ingrowth in ½ of the material
3. Ingrowth in ¾ of the material
4. Infiltration of tissue into the whole area of the foam
-. Disappearance of foam structure

Macrophages/foreign body giant cells (FBGCs)/ cells with phagocytic activity (WFA)

0. Hardly any cell present
1. Low number of cells
2. Low to medium number of cells
3. Medium number of cells
4. High number of cells

Light Microscopy (LM) degradation

0. Signs of degradation at the border of the material
1. Degradation in ½ of the material
2. Degradation in the centre of the material
3. With partly loss of porous structure
4. Intracellular remnants of material
5. Signs of cellular activity indicative of degradation without visible material
6. No signs of cellular activity indicative of degradation

Stage of tissue response and degradation

Encapsulation and infiltration (E/I)
Degradation and fragmentation (D/F)
Resorption (R)

weeks, intracellular remnants of collagen were still visible. Gelatin showed no biodegradable products after 26 weeks although there were some areas with increased phagocytic activity indicative for the presence of biomaterial. After 39 weeks, no signs of cellular activity indicative of degradation were seen in the gelatin samples. The collagen implants at 39 weeks showed increased cellular activity indicative for the presence of biomaterial. Beyond one year, no signs of cellular activity indicating degradation were seen in the collagen samples.

The phagocytosis of PU particles that was first seen at the one year time interval was still visible after 2.5 years. At the longest time interval of 3 years, PU implants showed areas with phagocytic activity without visible remnants of biomaterial. Although the PU was not detectible in these samples, the presence of macrophages is indicative for the presence of biomaterial. Thus, the PU definitely shows a slower degradation than gelatin and collagen.
The degradation of PU had progressed further in the rabbits than in the rats at the 1 year interval. Fragmented pieces of PU were seen in some of the rats after 1 year, whereas in the

rabbits only intracellular remnants of PU were detectible at this same time interval. At 1.5 years intracellular remnants of PU were seen in both rats and rabbits. This was characterized histologically by macrophages that phagocytised PU particles (Figure 5 and 6).

Renal functions

Renal functions of the animals were determined by measuring the blood urea nitrogen (BUN) and the creatinine concentration when the animals were sacrificed. These analyses were to determine whether implantation of small amounts of PEG would lead to deterioration of the renal function. Table 1 presents an overview of the mean concentrations of urea and creatinine per time interval. The mean weight at the time of sacrifice and the number of animals per time interval is also noted. In the rats a total of 0.006 or 0.017 grams of PEG was implanted, for the rabbits this was 0.011 or 0.022 grams. The amount was dependant on the implantation of the three different test materials in the animals or solely PU.

The normal blood values for BUN and creatinine in Wistar rats are 2.9–9.8 mmol/L and 35–332 µmol/L respectively [14]. The urea levels of all rats stayed within the normal range except for one of the rats after 1.5 years who slightly exceeded the maximum value (9.9 mmol/L). The values for creatinine stayed within or below the normal range. In New Zealand White rabbits the normal blood values for BUN are 2.9–8.9 mmol/L and the normal blood values for creatinine are 12–147 µmol/L [15]. These values were not exceeded in any of the rabbits.

Discussion

This implantation study was undertaken to examine the tissue-response and to compare the degradation processes and degradation time of PU foam to commonly used hemostatic agents like collagen and gelatin. To acquire a more rapid degradation process a high percentage of PEG was introduced in the composition of the PU foam. We found that the degradation time of PU did not match that of gelatin and collagen. It took 3 years before particles of PU implants were no longer detected. Gelatin and collagen particles were not seen after respectively 26 weeks and 39 weeks. This showed that the degradation process of the PU implant lagged considerably compared to collagen and gelatin. Furthermore, the degradation of gelatin seems to go somewhat faster than the degradation of collagen.

All three biomaterials followed a corresponding degradation pattern of encapsulation/infiltration, degradation/fragmentation and ultimately resorption. Therefore, a comparison between the three biomaterials could be made.

In the *in vivo* study by Van Minnen et al. [6] macrophages containing phagocytosed PU were found after 3 years of implantation. The porous structure of PU without PEG was mostly intact after 26 weeks, and this structure only started to disappear from 39 weeks onward [6]. In comparison to this study, minor differences in degradation time were noticed. In our study, macrophages containing phagocytosed biomaterial were not found after 3 years but were

seen up to 2.5 years. Also, the porous structure of PU with 55 wt% PEG started to disappear from 26 weeks onward. Therefore, it seems that the degradation of the current PU with 55 wt% of PEG occurs more rapidly than the degradation of PU without PEG. This small difference could be attributed to the PEG as this increases hydrophilicity and thus increases initial degradation rate [8,9]. The complete degradation of the remaining PU moieties by macrophages did not progress substantially faster as the process still took up to 2.5 years. Another difference in our study compared to the study by Van Minnen et al. [6] is that we introduced an extra degradation level in which signs of cellular activity indicative of degradation were seen, without visible material. This level indicates that some biomaterial particles might still be present but are too small to be detected with light microscopy. This level of degradation was seen in the PU samples after 3 years. We estimate that it might take up to 3.5 or 4 years before signs of degradation are not longer detectable anymore in areas where PU was implanted.

An *in vitro* study by Fromstein et al. showed a rapid initial degradation of PU with a mass percentage of 50% of polyethylene oxide (PEO), followed by prolonged degradation in the later stages [9]. This corresponds to our results as we used a comparable percentage of the nearly identical polyethylene glycol (PEG) in our PU foam.

Earlier studies have described the degradation period of gelatin and collagen to vary from 4-6 weeks and 6-12 weeks respectively [12,16]. The degradation of these materials is primarily accounted for by enzymes, such as gelatinase, collagenase and telopeptide cleaving enzymes [17,18]. In our study, intracellular remnants of gelatin were seen up to 12 weeks after implantation whereas collagen was traceable up to 26 weeks after implantation. We do not have a clear explanation for this difference but it could be contributed to the relatively small animals that were used in our study. The enzymatic activity in these smaller animals might be lower than in larger animals. We already assessed that the degradation process progressed faster in the rabbits than in the rats. Earlier studies were done with larger subjects like dogs, pigs and humans [19-21].

The degradation duration of PU in humans may therefore possibly be less than 2.5 years. A comparable study by Alpaslan et al. in which gelatin and collagen were subcutaneously implanted in rats showed that the materials had decreased in size and were surrounded with fibrous tissue 45 days after implantation [22]. In our study the degradation seemed to have progressed somewhat faster after 4 weeks as the material definitely showed signs of fragmentation.

Other studies investigated the properties and possibilities to increase the degradation rate of PU for applications as neural conduits or bone replacements [23,24]. This showed that alteration of the molecular structure of PU could influence the rate of hydrolysis, which plays a major role in biodegradable polymers [25]. These alterations focused on the soft segments like PEO which increases hydrophilicity and hygroscopy of PU, thus initiating degradation. However, when PU is combined with high amounts of PEO the material becomes weak and tacky [26]. Our PU foam was combined with the maximum concentration of 55 wt% PEG which is similar to PEO. A higher percentage of PEG would lead to insufficient mechanical

properties. However, this modified PU foam with a high percentage of PEG did not result in the desired duration period of degradation similar to gelatin and collagen. The material did show an earlier disintegration compared to PU without PEG [6]. The time to complete degradation however, still took up to 2.5 years. A faster PU resorption would be favorable because the material could lead to secondary inflammation while its presence as a hemostatic agent is no longer necessary. A possibility to further increase the rate of degradation could be found in the alteration of the hard segments. This has been done by Skarja et al. who specifically designed enzyme sensitive linkages in the hard segment of the PU [26,27].

The biocompatibility of PU with 55 wt% PEG has shown no severe inflammatory or foreign body response during the complete process of degradation. This is in accordance with other studies that analysed the biocompatibility of PU [6,25].

The possible toxicity of PEG was also examined in this study. To measure toxicity, blood samples were taken prior to termination for analysis of renal parameters blood urea nitrogen (BUN) and creatinine. These values stayed below or within the normal range. However, after 1.5 years the BUN values in rats increased near the maximum normal value. One of the rats even slightly exceeded this value. An increase of these parameters towards the maximum normal value has been described earlier in healthy rats as the age increases [28]. Based on these results, it is not expected that the implantation of PU with 55 wt% PEG will lead to nefrotoxicity in humans. Furthermore, the relative amount of PEG in humans would be considerably lower than the amount of PEG we implanted in rats and rabbits. An explanation for the relatively low creatinine levels in rats may be the lack of exercise.

In this degradation study we chose to implant the biomaterials in animals because this would provide us with the most data and reliable *in vivo* information. The materials were implanted in both rats and rabbits as the life span of rats would not exceed the three year term, chosen for this study. Implantation of the biomaterials in rabbits only, was not practicable for logistic and financial reasons. Although the rate of degradation was a little faster in rabbits, the overall process of degradation could be followed accurately.

The choice, to sacrifice rats with several successive short term time intervals at the beginning of the study, was made to follow the degradation process of the rapid degrading abilities of gelatin and collagen more accurately [22].

The rapidly changing stages of degradation of collagen and gelatin after only 2 weeks justify the choice of short term time intervals. The PU implants also showed a change in degradation after 1 and 2 weeks. This change in different levels of degradation could indicate that the initial degradation was induced by the high percentage of PEG.

A semiquantitative scoring method was needed to obtain an overall representation of the degradation processes of all biomaterials in time. At the later time intervals, relocating the biomaterial became more difficult. The explanted tissue might therefore not also have been the center of the degradation area. Due to the large number of implants and the gradual pattern of degradation we still could acquire a reliable impression of biodegradation of PU, collagen and gelatin.

Conclusions

The degradation process of PU, collagen and gelatin follows a gradual and comparable pattern towards resorption. However, the time to complete resorption is considerably faster for collagen and gelatin than for PU. The biocompatibility of all biomaterials was amply sufficient and no inflammatory reaction or severe foreign body response occurred. The biocompatibility and predictability of degradation indicate that PU can be used as a topical hemostatic agent but a more rapid degradation would be favorable. Future research should therefore aim on PU foam with the desired degradation rate of the polyurethane moieties yet maintaining its hemostatic and viscoelastic properties.

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Chapter 7

Risk of bleeding after dentoalveolar surgery in patients taking anticoagulants

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Abstract

To avoid increasing the risk for thromboembolic events, it is recommended that treatment with anticoagulants should be continued during dentoalveolar operations. We evaluated the incidence of bleeding after dentoalveolar operations in a prospective study of 206 patients, 103 who were, and 103 who were not, taking anticoagulants. Seventy-one were taking thrombocyte aggregation inhibitors (TAIs) and 32 with vitamin K antagonists (VKAs). Patients were treated according to guidelines developed at the Academic Centre for Dentistry Amsterdam (ACTA), the Netherlands. The patients underwent surgical extraction, non-surgical extraction, apicectomy, or placement of implants. They received standard postoperative care and patients taking VKAs used tranexamic acid mouthwash postoperatively.

No patient experienced severe bleeding requiring medical intervention. Seven patients (7%) on anticoagulant therapy suffered mild post-operative bleeding. Patients taking VKAs reported three mild bleeding incidents (9) compared with 4 (6%) in the group taking TAIs. Among patients without anticoagulant therapy, two (2%) suffered mild bleeding. The between-group differences were not significant. All bleeding incidents were controlled by the patients themselves with compression with gauze.

We conclude that dentoalveolar surgery is safe in patients being treated with anticoagulants provided that the conditions described in the ACTA guidelines are met.

Introduction

Oral anticoagulant therapy is common and has been proved to be effective in preventing thromboembolic events [1,2]. The most commonly used agents are thrombocyte aggregation inhibitors (TAIs; like acetylsalicylic acid and clopidogrel) and vitamin K antagonists (VKAs; like warfarin, acenocoumarol, and fenprocoumon).

As patients increasingly tend to keep their natural dentition up to higher ages, dentoalveolar surgery is more frequently indicated for elderly people using anticoagulant therapy. Some health care providers still instruct all patients to discontinue anticoagulant therapy before dentoalveolar surgery, regardless of the individual thromboembolic risks of the patient. However, this can result in thromboembolic events (such as deep vein thrombosis and pulmonary embolism) that are worse than postoperative bleeding after dentoalveolar surgery [3].

The Academic Centre for Dentistry Amsterdam (ACTA) of the Netherlands developed guidelines based on the publications of van Diermen *et al.* [4-6]. These guidelines recommend that anticoagulants should be continued during dentoalveolar surgery under well-described conditions [7]. These guidelines are accepted by the professional organization for dentists in the Netherlands (NMT) and the organizations charged with ambulant antithrombotic treatment, and are used by most Dutch oral health care professionals.

The guidelines make a distinction between the two types of anticoagulants. For both TAIs and VKAs accounts that the dentoalveolar surgery should involve no more than three extractions at the same time, surgical removal of wisdom teeth, periodontal treatment, apicectomies, abscess incision, or the placement of a maximum of three implants. Patients using one TAI can continue this agent during dentoalveolar surgery without further restrictions. In contrast, patients on VKAs can continue these agents only when a number of specific conditions are met. The international normalized ratio (INR), measured within 24–72 hours preoperatively, must be $\leq$3.5. Furthermore, the operation must be performed as atraumatically as possible, the wound must be sutured after extraction, and the patient should leave the hospital with adequate instructions only once the bleeding has stopped. Finally, the patient should rinse with tranexamic acid 5% for five days postoperatively [7].

In the present study we evaluated the ACTA guidelines to find out whether these procedures led to increased postoperative bleeding. We compared the incidence of postoperative bleeding in patients on anticoagulant therapy with that in a group of patients using no coagulation-altering medication.

Patients and Methods

Subjects

The study group comprised 206 patients who were referred to the Department of Oral and Maxillofacial Surgery of the University Medical Center Groningen, the Netherlands, for a type of dentoalveolar surgery meeting the ACTA guidelines, i.e., with a maximum of three extrac-

Table 1. Characteristics of patients and distribution of postoperative bleeding per type of procedure.

	Anticoagulant (n=103)	**No anticoagulant (n=103)**	**Total (n=206)**
Sex Male/female	75/28	68/35	143/63
Mean age in years at time of treatment (range)	62 (23-86)	56 (21-85)	59 (21-86)
Surgical extractions (mild bleeding)	48 (5)	55 (1)	103 (6)
Non-surgical extractions (mild bleeding)	42 (2)	37 (1)	79 (3)
Apicectomies (mild bleeding)	5 (0)	4 (0)	9 (0)
Implant placement (mild bleeding)	8 (0)	7 (0)	15 (0)

tions, apicectomies, or implant placements performed at the same time. The types of dento-alveolar surgery required were divided into four categories: surgical extractions, non-surgical extractions, apicectomies, and implant placement. Extractions were defined as surgical when the surgeon had to incise the gingiva before extraction.

Of the 206 included patients, 103 used oral anticoagulant therapy (therapy group) and 103 did not use any anticoagulant therapy (control group). Patients on VKAs were included if their INR, measured within 72 hours prior to surgery, was in the range from 1.8–3.5. Patients on TAIs were included if they used only one TAI.

Exclusion criteria were inherited or acquired coagulopathy from systemic. Additionally, patients in the control group were excluded if they used any coagulation-altering medication. The study was conducted according to the Declaration of Helsinki, and informed consent was obtained from all participants.

Study protocol

In this prospective study, patients were treated according to the ACTA guidelines. Before the procedure, all patients received a local anesthetic (4% articaine with 1:100,000 epinephrine). The operation was performed as atraumatically as possible and the wound was sutured after surgery. After extraction, patients were instructed orally and in writing to apply gauze compression for 30 minutes immediately after the operation. Patients were not allowed to leave the hospital before the bleeding had stopped, and were instructed to call the department of oral and maxillofacial surgery if the bleeding did not stop after 30 minutes. The 32 patients who used VKAs were instructed to rinse with 5% tranexamic acid four times a day for five days postoperatively.

Table 2. Number (%) of incidents of postoperative bleeding.

	None (n=197)	Mild bleeding (n=9)
Anticoagulant (n=103)	96 (93%)	7 (7%)
No anticoagulant (n=103)	101 (98%)	2 (2%)

There were no episodes of severe bleeding.

Two types of postoperative bleeding were defined. If the patient came to the hospital because the bleeding could not be stopped at home, it was scored as severe. At one week postoperatively, all included patients were called by a researcher to ask if postoperative bleeding had occurred that was stopped at home by compression with gauze; such bleeding was scored as mild.

Statistical analysis

Data were analysed using SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as mean and standard deviation (SD). Continuous variables were assessed using the Student's *t*-test, and dichotomous variables using the Pearson's chi-squared or Fisher's exact test, as appropriate. Linear regression analysis was used to determine the relationship between INR value and mild bleeding incidents. A p value of <0.05 was considered statistically significant.

Results

Of the total 206 included patients, 143 were male and 63 were female, and the mean age was 59 years (range, 21–86 years) at the time of treatment. Table 1 summarizes the patient characteristics and the types of surgery that were performed.

The difference between the sexes was not significant ($p=0.29$), while the difference in mean age at the time of treatment was ($p<0.01$). The most frequently performed procedures were surgical and non-surgical extractions, and all bleeding incidents occurred in patients who underwent these procedures. No postoperative bleeding incidents were recorded in patients who underwent apicectomies or implant placements.

Bleeding incidents were most common in patients who underwent a surgical extraction while they were on anticoagulant therapy. However, this incidence was not significantly different from that in patients who underwent non-surgical extractions while on anticoagulant therapy ($p=0.4$). Among patients who did not use anticoagulant therapy, the groups that underwent surgical extractions and non-surgical extractions each reported one postoperative bleeding incident. Although the non-surgical group was smaller, the between-group difference in incidence was not significant ($p=1.0$).

Table 3. Number (%) of mild postoperative bleeding incidents by type of anticoagulant.

	No bleeding	Mild bleeding
Thrombocyte aggregation inhibitors (n=71)	67 (94%)	4 (6%)
Vitamin K antagonists (n=32)	29 (90%)	3 (9%)
No anticoagulant treatment (n=103)	101 (98%)	2 (2%)

There were no episodes of severe bleeding.

Regarding the major groups of patients with and without anticoagulant treatment, table 2 presents the number of postoperative bleeding incidents per group.
None of the included patients experienced severe postoperative bleeding. Patients without anticoagulant therapy experienced a lower number of mild bleeding incidents than the group with anticoagulant treatment; however, the difference between these groups was not significant (p=0.17).

Table 3 shows the data according to the different types of anticoagulant therapy. The group of patients that used VKAs had the highest percentage of mild postoperative bleeding incidents, and had a mean (range) INR of 2.6 (1.9–3.4). The 3 patients that suffered from a mild bleeding incident had an INR of 2.0, 2.9 and 3.0. Linear regression analysis revealed that the INR value was not correlated with the occurrence of mild bleeding incidents (β=0.41, SE=0.28, p=0.14). Patients using VKAs had a non-significantly higher incidence of mild postoperative bleeding compared to patients that used TAIs (p=0.67) and patients who did not use anticoagulants (p=0.09). Patients using no anticoagulant therapy had the lowest incidence of bleeding, but the difference between this group and the patients that used TAIs was also not significant (p=0.23). Figure 1 presents an overview of the percentages of mild bleeding incidents in the different groups.

Discussion

Continuation of anticoagulant therapy can be extremely important for patients with a high thromboembolic risk, such as patients with mechanical heart valve prostheses and recurrent or recent thromboembolic events. When planning dentoalveolar surgery in such patients, the possible consequences of postoperative bleeding in patients on continuous anticoagulant therapy must be weighed against the possible consequences of a thromboembolic event. To help guide such decisions, here we analysed the frequency and severity of postoperative bleeding after dentoalveolar surgery in patients with and without anticoagulant therapy.
None of the patients in our study exhibited severe postoperative bleeding. This is in accordance with the results of Napenas *et al.*, who found no postoperative bleeding complications

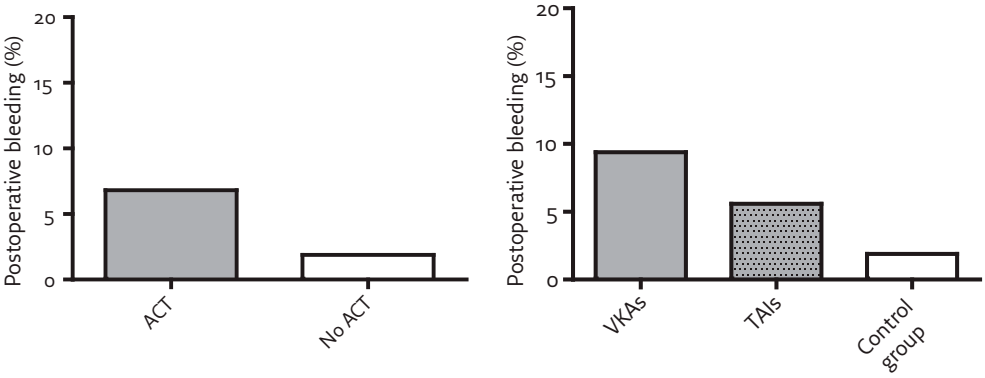

Figure 1. Percentage of mild postoperative bleeding incidents for the different groups. ACT, anticoagulant therapy; VKAs, vitamin K antagonists; TAIs, thrombocyte aggregation inhibitors.

that resulted in visits or phone calls among 43 patients receiving single or dual antiplatelet therapy; however, that study did not follow-up with patients after surgery to record mild postoperative bleeding incidents [8].

In our study, we called the patients one week after surgery and found that 6.8% of the patients on anticoagulant therapy suffered from a mild postoperative bleeding. In a similar study by Brennan *et al.*, patients using one TAI were called two days after dentoalveolar surgery; of 14 patients, 2 (14%) reported bleeding that stopped after applying gauze compression at home [9]. This difference in incidence might be explained by the relatively low number of patients in the latter study, as one postoperative bleeding incident leads to a considerable percentage fluctuation.

Other studies evaluating the safety of continuing of anticoagulant therapy during dentoalveolar surgery have recorded prolonged bleeding time or the severity of intraoperative bleeding. Lillis *et al.* reported prolonged immediate bleeding after extraction in 2 out of 78 patients (2.6%) taking one TAI, and in 2 out of 532 patients (0.4%) without anticoagulant therapy; this difference was not statistically significant [10]. In accordance with our study, they reported no late bleeding complications among their patients. Ardekian *et al.* examined the severity of intraoperative bleeding during dentoalveolar surgery [11]. Among the 19 patients that used one TAI, 4 (21%) experienced severe intraoperative bleeding, which was a significantly higher incidence than that found in patients using no anticoagulant therapy (10%). All bleeding could be controlled by suturing the wound and applying pressure with gauze, and no uncontrolled postoperative bleeding was reported during the week after surgery.

In the present study, we chose to record the frequency and severity of postoperative bleeding *after* dentoalveolar surgery because we feel that this is the most clinically relevant parameter. Unlike most studies that have analysed the safety of continuing anticoagulant therapy during dentoalveolar surgery, we contacted all patients one week postoperatively to record whether bleeding had occurred at home. This method enabled us to also analyse the mild bleeding in-

cidents, and any severe bleeding incidents that might have been treated by another hospital, which would go unnoticed in studies that only record whether a patient seeks contact after dentoalveolar surgery.

For the therapy group in this study, we included all patients who met the conditions described in the ACTA guidelines. Because these guidelines address both the use of TAIs and VKAs, our population included patients that used either of these therapies. This enabled us to compare the percentage of postoperative bleeding according to the different anticoagulant therapies. We found that the VKAs led to a higher percentage of mild bleedings, although this difference was not significant. However, any comparison between these treatment groups should be interpreted cautiously; TAIs are prescribed more often, and thus the study included a higher amount of patients that used this type of medication.

The ACTA guidelines were modified in 2013, and now state that dual TAI therapy can also be continued during dentoalveolar surgery. As the subjects in our study were recruited before 2013, patients on dual TAI therapy were not included.

According to the ACTA guidelines, the antifibrinolytic agent tranexamic acid is indicated in patients that use VKAs. Patients that used a TAI or who did not use anticoagulant medications did not receive treatment with tranexamic acid or other topical hemostatic agents. This was in accordance with other studies that have only used topical hemostatic agents in cases of postoperative bleeding or prolonged bleeding time [10,12]. However, there is no available evidence-based protocol for determining when topical hemostatic agents are indicated during or after dentoalveolar surgery.

The composition of the presently studied population could have influenced the results in several ways. All mild postoperative bleeding incidents occurred in patients who underwent surgical or non-surgical extractions, while none were seen in patients that underwent implant placement or apicectomies. This might be due to the considerably lower number of patients that underwent implant placement or apicectomies. Another reason could be that the wound can be closed primarily after these operations, which is not always possible after (surgical) extractions.

The baseline characteristics were not entirely equal between the groups with and without anticoagulant therapy, with a significant difference found in mean age at the time of treatment. This was due to the fact that elderly patients more often use anticoagulant therapy and therefore were more often included in the therapy group. We estimate that this difference in mean age did not influence the results because the bleeding tendency in the control group is not expected to be different when the mean age would have been higher.

Conclusions

The present study analysed the frequency and severity of postoperative bleeding after dentoalveolar surgery in patients with and without anticoagulant therapy. None of the patients

suffered from severe postoperative bleeding. Mild postoperative bleeding incidents that were stopped by the patients themselves occurred more often in the patients on anticoagulant therapy; however this difference between groups was not statistically significant. Therefore, we conclude that it is safe to perform dentoalveolar surgery without stopping anticoagulant therapy, provided that the conditions described in the ACTA guidelines are met.

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Chapter 8

Summary and general discussion

Summary

A broad range of topical hemostatic agents is available to the surgeon in case of problematic bleeding. Most of these hemostatic agents are based on animal derived products like collagen and gelatin. As these agents carry the potential risk of pathogen transmission, a fully synthetic hemostatic agent with at least a comparable efficacy could replace these agents.
In this thesis, multiple studies on the hemostatic efficacy, mechanical properties and degradation characteristics of synthetic polyurethane foam (PU), based on butane-diisocyanate, and with 55 wt% polyethylene glycol (PEG), are described. Furthermore, the incidence and severity of bleeding incidents after dentoalveolar surgery has been assessed.

In **chapter 2** the PU foam was tested *in vitro* and compared to collagen and gelatin hemostatic agents to analyse if the hemostatic efficacy of these agents could be matched. A test model was developed in which human whole blood flowed through the test materials. The blood flow deceleration was determined as a derivative for the extent of hemostasis. Several modifications were applied to the PU to evaluate if this would increase its hemostatic efficacy. The addition of recombinant factor VIIa, phospholipids, adenosine diphosphate and thrombin gave no improved results. However, treatment of the PU foams with glow discharge, which increased the hydrophilicity of the material, led to increased hemostasis. Increasing the PEG concentration of the PU to 55 wt%, and therewith increasing the hydrophilicity of the material, also showed improved results that were similar as gelatin. The best results in this study were achieved with collagen; these results could not be matched by PU.
From this study it was concluded that increasing the PEG concentration of the PU is a promising approach. Therefore, the applicability of PU with 55 wt% PEG as a topical hemostatic agent was studied in the subsequent chapters.

As hemostasis is a complex process which is difficult to simulate *in vitro*, it was plausible to study the hemostatic efficacy of PU in an *in vivo* model. In **chapter 3** a human split-mouth model was used to assess the hemostatic efficacy of PU with 55 wt% PEG. In this study we included 60 patients who had to undergo extraction of an upper and lower molar. After extraction of a molar, a PU foam and collagen or gelatin agent were inserted in separate extraction sockets for 2 minutes. At a later stage, the concentration of coagulation parameters thrombin-antithrombin III (TAT) complexes, fibrinogen, and thromboxane B_2 (TxB_2) in blood extracts from the agents was measured. The concentrations were also determined in baseline blood samples which were collected from the extraction socket. In all materials the concentrations of TAT and TxB2 were significantly increased, and fibrinogen concentration was significantly reduced compared to baseline wound blood concentrations indicating increased hemostasis supplementary to the activation induced by the wound area. No significant differences were seen in these concentrations of the coagulation parameters in the three different hemostatic agents. These results suggest that the hemostatic efficacy of PU is

equal to collagen and gelatin and thus forms a promising alternative for these animal-derived hemostatic agents.

In **chapter 4**, we compared the hemostatic efficacy of PU with other hemostatic agents in a rat tail-tip model. This study was performed to obtain an overview of the differences in time to hemostasis between the most widely used topical hemostatic agents. Furthermore, we tested PU enriched with the procoagulant substance chitosan to evaluate if this could increase its hemostatic efficacy.

In 80 rats a similar wound was created at the tail tip which was coagulated with a hemostatic agent. The tail tip was fixed on a developed test device to ensure a constant and equal pressure of the test material on the wound. The mean bleeding time was determined and compared between the groups. PU with chitosan showed the shortest mean bleeding time. This was significantly shorter than collagen, oxidised regenerated cellulose and chitosan dressing. The difference between PU with and without chitosan was not statistically significant. Our findings suggest that the hemostatic efficacy of PU is equal to the most widely used hemostatic agents. The addition of a procoagulant substance like chitosan showed a tendency towards shorter bleeding times but did not lead to a significantly faster hemostasis.

In **chapter 5**, we investigated the mechanism of hemostatic action of PU and compared this to collagen and gelatin. Furthermore, the viscoelastic properties of these materials were assessed. The hemostatic action mechanism of the materials was tested by analysing the effect on thrombocyte aggregation in human whole blood and measuring the coagulation time of platelet-poor plasma. The ability of the hemostatic agent to exert pressure on the wound was quantified in terms of its viscoelastic properties both under dry and wet conditions using a low load compression tester. The low amount of free thrombocytes in the blood samples of PU and collagen indicates that these materials lead to a high amount of aggregated and adhered thrombocytes. The amount of free thrombocytes in the blood samples of gelatin showed no significant difference from the control group. The mean coagulation times of the platelet-poor plasma samples that were incubated with PU and collagen were significantly lower than in the control samples without material, indicating that these materials initiate the coagulation cascade. The mean coagulation time of the samples that were incubated with gelatin did not differ significantly from the control samples without material. Analysis of the viscoelastic properties revealed that the deformation under wet conditions of collagen and gelatin is higher than the deformation of PU. Furthermore, the percentage of stress relaxation was lowest for PU followed by collagen and gelatin indicating that PU has the highest springback capability. From this study we concluded that PU and collagen initiate hemostasis through both thrombocyte aggregation and contact activation of the coagulation cascade whereas gelatin did not show improved thrombocyte aggregation or initiation of the coagulation cascade. PU has favorable viscoelastic properties compared to collagen and gelatin, which leads to better handling capacities and enhanced pressure on a wound.

The degradation process and time of PU was studied and compared to collagen and gelatin in **chapter 6**. The materials were implanted subcutaneously in rats and rabbits that were sacrificed at certain time intervals up to three years. Renal functions of the animals were determined to analyse the possible nefrotoxicity of PEG. The materials, which were explanted at the different time intervals, were evaluated with light microscopy. This examination showed a comparable pattern of degradation for the test materials. However, the degradation process was completed remarkably faster for collagen and gelatin than for PU as PU particles were detectible in the samples up to 2.5 years. All of the materials showed a good biocompatibility and no inflammatory reactions or severe foreign body reactions occurred. Mean urea and creatinine values stayed within the normal range during the three year investigation period indicating that the implanted amount of PEG does not lead to nefrotoxicity. Based on these results we conclude that PU can be used as a topical hemostatic agent although a shorter degradation period would be desirable.

In **chapter 7**, we evaluated the bleeding incidence after dentoalveolar surgery in patients with and without anticoagulant therapy. We included 206 patients: 103 without anticoagulant therapy and 103 with anticoagulant therapy [71 with thrombocyte aggregation inhibitors (TAIs) and 32 with vitamin K antagonists (VKAs)]. Patients were treated according to a guideline developed at the Academic Centre for Dentistry Amsterdam (ACTA), the Netherlands. The patients underwent surgical extraction, non-surgical extraction, apicoectomy, or implant placement. They received standard postoperative care and patients taking VKAs used tranexamic acid mouthwash postoperatively which has an antifibrinolytic effect. None of the patients experienced severe bleeding requiring medical intervention. In the anticoagulant therapy group, seven patients (6.8%) suffered from a mild post-operative bleeding. In the control group without anticoagulant therapy, two patients (1.9%) experienced mild post-operative bleeding. These mild post-operative bleeding incidents could be controlled by the patients themselves with compressive gauze. From this study it was concluded that it is safe to perform dentoalveolar surgery in patients receiving anticoagulant therapy, provided that the conditions described in the ACTA guideline are met.

General discussion

Hemostatic efficacy is the most important property of a topical hemostatic agent. Additional properties are handling characteristics, bioresorption potential, absence of antigenic response, and tissue reactivity effects. The studies described in this thesis demonstrate that a synthetic topical hemostatic agent based on polyurethane (PU) comprising polyethylene glycol (PEG) has favorable hemostatic, mechanical and tissue reactivity properties. The bioresorption potential is promising although the degradation time is remarkably longer than the currently used topical hemostatic agents.

Hemostatic efficacy of polyurethane foam with 55 wt% polyethylene glycol

The *in vitro* analysis on hemostatic efficacy showed that PU foam has hemostatic potential although the results of collagen could not be matched (**chapter 2**). In **chapter 5**, the mechanism of hemostatic action of PU, collagen and gelatin was investigated *in vitro* by evaluating the effect on thrombocyte aggregation and the coagulation cascade. In this study, collagen and PU both showed a comparable effect on thrombocyte aggregation but collagen showed a faster initiation of the coagulation cascade. In both *in vitro* studies, the results of gelatin fell short compared to collagen and PU. The good results of PU in these studies could be attributed to the high concentration of PEG as this increased the absorbability and hydrophilicity of the material. The absorbable properties might enhance concentration of the endogenous coagulation factors and platelets as the effect of cellulose- and polysaccharide-based hemostats is also based partly on this mechanism [1,2]. Increasing the hydrophilicity of polyethylene has also shown to enhance platelet adhesion and activation of the clotting system [3]. The good hemostatic properties of the PU foams that were treated with glow discharge in **chapter 2** are a further indication that increasing the hydrophilicity of the material leads to a better hemostatic efficacy. The use of PEG in the form of hydrogels has already been successfully used in surgery to accomplish hemostasis [4,5]. Its mechanism of action in this form depends on mechanically sealing the areas of blood leakage [6].

The good results of collagen in these *in vitro* studies can be largely explained by its action mechanism which is based predominantly on platelet aggregation. The collagen attracts platelets when they get into contact with blood. The platelets adhere to collagen fibrils and degranulate, thereby triggering platelet aggregation [7,8]. This does however not explain the short coagulation time of platelet poor plasma after it was incubated with collagen in **chapter 5**. Our findings suggest that the hemostatic efficacy of collagen also arises out of initiation of the coagulation cascade. This has been described earlier in the literature when it was shown that factor XII can be activated by collagen in platelet poor plasma and thus can lead to coagulation through initiation of the coagulation cascade [9].

The poor results of gelatin are remarkable as it is a commonly used hemostatic agent with good hemostatic results [10]. The action mechanism of gelatin is posed to be mostly mechanical and seems likely to involve physical surface effects rather than any action on the blood clotting mechanism [7,8]. A previous *in vitro* study also showed very low platelet deposition for gelatin when the material was exposed to platelet rich plasma [11]. The poor results of gelatin could therefore indicate that the complexity of the coagulation cascade was not adequately imitated in these *in vitro* studies.

In our human *in vivo* study (**chapter 3**), hemostatic efficacy was examined by measuring different coagulation parameters in a split-mouth model. Between the PU foam and gelatin- or collagen-based materials no significant differences were seen in these coagulation parameters.

The significant differences between the baseline blood values and the values in the blood

extracts from the materials showed that all tested materials increased hemostasis. The coagulation cascade was already initiated in the baseline samples as these were taken from a wound [12]. The measurement of thrombin-antithrombin III (TAT) complexes and fibrinogen gave us an indication of the additional effect of the materials on the coagulation cascade. At the end stage of this cascade, fibrinogen is converted into fibrin under the influence of thrombin [12]. The increase in TAT complexes and reduction of fibrinogen compared to baseline values showed that PU, gelatin and collagen substantially increased hemostasis through the coagulation cascade. Thromboxane B_2 (TxB_2) is an inactive metabolite of thromboxane A_2 which can be used to quantify platelet aggregation [13]. The high TxB_2 values in the blood extracts from the tested materials show that they also have a substantial effect on platelet aggregation. The good *in vivo* results of gelatin, compared to the *in vitro* studies, show that this material does perform a functional role in hemostasis which apparently is difficult to measure *in vitro*. This was confirmed in **chapter 4** where we described an animal *in vivo* study on the hemostatic efficacy of PU compared to the most widely used topical hemostatic agents. In this study we also observed only minor differences in hemostatic efficacy between the topical hemostatic agents.

Gelatin and collagen are together with oxidized regenerated cellulose traditionally the most widely used hemostatic agents in surgery [8]. Oxidized regenerated cellulose was not tested in **chapter 3** because the structure of the material made it impossible to extract plasma samples that could be used for measurement of the coagulation parameters. In **chapter 4** the oxidized regenerated cellulose could be included together with gelatin, collagen and a chitosan dressing to be compared with PU. The relatively equal results of the materials in **chapter 3** and **chapter 4** are consistent with other studies that could not demonstrate differences in hemostatic efficacy between these widely used hemostatic agents [14-16]. There are however some studies that designated collagen as the most effective topical hemostatic agent which corresponds to our findings in **chapter 2** [11,17]. Other studies found chitin to be the most effective topical hemostatic agent [18,19]. These studies and other reports on the good hemostatic efficacy of the chitosan dressing HemCon [20,21] led to the decision to include this material in the study described in **chapter 4** and to analyse if the addition of chitosan to PU could increase its hemostatic efficacy. The addition of chitosan could not significantly improve the hemostatic efficacy of PU which could be caused by mixing the chitosan through the foam. As a result the chitosan concentration at the surface of the foam might not have been high enough to exert an additional effect. The chitosan dressing HemCon showed a bleeding time that was significantly longer than gelatin and PU with chitosan. This discrepancy from earlier studies might be explained by the hemostatic action mechanism of HemCon. This material becomes sticky upon contact with blood and creates an enhanced tissue adhesion [22]. This working mechanism was probably disadvantageous in the used test model because the material had to be removed from the wound regularly to analyse if the wound was dry. The sticky property of the chitosan could have led to partial removal of the blood clot and thus a longer bleeding time.

Besides the surface reaction of a hemostatic material with blood components, compression of a wound is known to be effective and important for hemostasis [23]. Therefore, it is important that a topical hemostatic agent keeps its firmness and shape after it is soaked with blood. The results in **chapter 5** showed that the mechanical stability of collagen and gelatin foam significantly decreased after getting soaked, whereas PU foam maintained its stability. Furthermore, PU showed the highest amount of springback and therefore will keep more pressure on a wound even after getting soaked with blood. Collagen and especially gelatin become mushy and formless under wet conditions and are therefore less likely to exert enough pressure on a wound. The more firm PU also ensures better handling capacities than the mushy and formless collagen and gelatin. We did not find any indications for possible compressive complications due to extreme swelling of gelatin and collagen which is often mentioned in the literature [4,8,24,25]. The poor viscoelastic properties of gelatin are probably due to the production process in which denaturation of collagen leads to breaking of the triple-helix structure [26]. The better viscoelastic properties of collagen compared to gelatin were also observed in another study [27].

Degradation characteristics of polyurethane foam with 55 wt% polyethylene glycol

Increasing the PEG concentration to 55 wt% and therewith increasing the hydrophilicity of the polyurethane led to a better hemostatic efficacy in **chapter 2**. Another advantage of the increased hydrophilicity is the presence of a greater volume of water within the polyurethane which allows for a greater amount of hydrolysis to take place [28,29]. In **chapter 6** it was demonstrated that the initial degradation of PU with a high percentage of PEG occurred more rapidly compared to PU without PEG [30]. However, the total degradation time was nearly identical with detectible PU remnants up to 2.5 years after implantation. A further increase in PEG will probably not lead to a faster overall degradation period and might lead to a weak and tacky material with inferior viscoelastic properties (**chapter 5**) [31]. Other studies have tried to increase the rate of degradation by inserting enzyme sensitive linkages in the hard segment of the PU [31-33]. This led to an increased susceptibility to enzyme-mediated but not buffer-mediated erosion when compared to a control PU [28].
The PU with 55 wt% PEG has shown no severe inflammatory, (nefro-)toxic or foreign body response during the complete process of degradation (**chapter 6**). This is in accordance with other studies that analysed the biocompatibility of PU [30,34]. The degradation periods of collagen and gelatin were considerably faster with remnants of gelatin seen up to 12 weeks after implantation and traceable remnants of collagen up to 26 weeks after implantation (**chapter 6**). These short degradation periods of collagen and gelatin are confirmed by the literature [8,35,36].

Postoperative bleeding in patients taking anticoagulants

Chapter 7 showed that anticoagulants can be safely continued in patients who need to

undergo dentoalveolar surgery. Prerequisite for this is that the recommendations from the ACTA guideline are followed. Important recommendations are the maximum value of 3.5 for the international normalized ratio (INR) and the postoperative use of the antifibrinolytic agent tranexamic acid in patients on vitamin K inhibitors. These recommendations are based on publications by van Diermen et al. [37-40] and are supported by several other studies [41-43]. Especially in oral surgery, patients might benefit from the use of tranexamic acid as the oral mucosa and saliva are rich in plasminogen activators [44]. The consensus that the oral anticoagulant regimen can be maintained in patients who undergo oral surgery is confirmed by this thesis (**chapter 7**) [45]. The use of other topical hemostatic agents like collagen and gelatin is not routinely recommended in patients taking oral anticoagulants but are mainly used in cases of postoperative bleeding or prolonged bleeding time [46,47]. The results from **chapter 7** showed that, when the recommendations from the ACTA guideline are followed, no severe postoperative bleeding incidents occurred which required medical treatment including the use of topical hemostatic agents. Mild postoperative bleeding incidents, which required compression with a gauze by the patients themselves, predominantly occurred in patients using oral anticoagulants who had to undergo one or more surgical extractions. There might be a role for topical hemostatic agents in preventing these mild bleeding incidents. Routinely inserting topical hemostatic agents in the extraction socket after surgical extraction in patients using anticoagulants could reduce the amount of mild bleeding incidents. Moreover, the continuation of oral anticoagulants during oral surgery is also advocated for patients receiving dual thrombocyte aggregation inhibitor therapy [48]. This could lead to a further increase of the incidence of mild and possible severe postoperative bleeding incidents [49].

Future perspectives

The studies in this thesis demonstrated that PU foam with 55 wt% PEG has a hemostatic efficacy that is comparable to the most widely used hemostatic agents. Hemostasis by the PU foam is induced by initiation of the coagulation cascade and platelet aggregation and its viscoelastic properties are favorable compared to collagen and gelatin. Significantly increasing the hemostatic efficacy of PU by the addition of procoagulant substances was not successful in the tests on this subject that were described in this thesis. However, we estimate that the addition of procoagulant substances should be able to improve the hemostatic efficacy of PU, since collagen and gelatin to which a procoagulant substance like thrombin has been added have shown an enhanced hemostatic efficacy [50].

The PU with 55 wt% PEG has shown to be a material that can be safely used as a biodegradable implant. Its degradation time could however not approach the degradation times of collagen and gelatin. The solution to this issue could be found in alteration of the hard segments of the polyurethane which are composed of butanediol and 1,4-butanediisocyanate. This could be done by introducing an enzyme sensitive linkage as has been described in other

studies [31-33]. Another option could be alteration of the chain length of the urethane moieties. The currently tested polymer was composed of 5 urethane moieties whereas a length of 3 urethane moieties might lead to a more rapid degradation period. Future research will have to show if these possible changes in the hard segments can increase the rate of degradation while maintaining the hemostatic and mechanical properties of the foam.

The number of patients on anticoagulant therapy that need to undergo dentoalveolar surgery will probably increase in the upcoming years as cardiac diseases are becoming more frequent in our aging society [51]. The role of hemostatic agents is not clearly established in these patients as can be deduced from the rapidly changing guidelines on this subject. The antifibrinolytic agent tranexamic agent will probably maintain a major role in patients using vitamin K inhibitors, although the effective concentration and the necessary length of postoperative use have yet to be determined.

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Nederlandse samenvatting

Lokale hemostatica zijn hulpmiddelen die ter plaatse van een wond de bloedstolling bevorderen. Ze kunnen worden gebruikt bij patiënten die een bloeding hebben als gevolg van een trauma of stoornis in de bloedstolling. Middelen om bloedingen te stoppen worden al duizenden jaren gebruikt.
Tegenwoordig worden lokale hemostatica in alle chirurgische disciplines gebruikt. De hemostatica zijn in verschillende soorten en maten beschikbaar. De meeste hemostatica zijn geproduceerd op basis van dierlijke materialen als collageen en gelatine. Een nadeel van deze materialen is dat ze in natte toestand snel hun mechanische eigenschappen verliezen en mogelijk ziekten kunnen overbrengen van dier op mens. Een synthetisch hemostaticum met goede visco-elastische eigenschappen op basis van polyurethaan (PU) en polyethyleenglycol (PEG) zou deze nadelen kunnen wegnemen. Daarnaast is het productieproces bij een synthetisch materiaal veel beter te controleren en waar nodig aan te passen. Het PU dat in dit proefschrift is beschreven is samengesteld uit niet-toxische componenten die afbreekbaar zijn door het menselijk lichaam waardoor het veilig gebruikt kan worden. Dit in tegenstelling tot bijvoorbeeld het polyurethaanschuim dat gebruikt wordt voor de isolatie van gebouwen.
Het doel van dit promotieonderzoek was te komen tot een klinisch toepasbaar synthetisch hemostaticum op basis van PU en PEG. Hiervoor is onderzocht of de hemostatische effectiviteit van het materiaal tenminste gelijk is aan de effectiviteit van de bestaande materialen. Verder is onderzocht hoe snel het materiaal oplost in vergelijking met collageen en gelatine. Ook is er een hoofdstuk opgenomen waarin de effecten op nabloedingen worden geëvalueerd van het doorgebruiken van anticoagulantia (bloedverdunners) tijdens een operatieve ingreep in de mond.
In **hoofdstuk 2** is een studie beschreven waarbij in een testopstelling in het laboratorium het PU schuim is vergeleken met collageen en gelatine. In deze testopstelling stroomde humaan (menselijk) bloed door de testmaterialen. De afname van de stroomsnelheid van het bloed werd bepaald als afgeleide voor de mate van bloedstolling. Er werden verschillende aanpassingen aan het PU gedaan om te evalueren of hiermee de hemostatische effectiviteit van het materiaal kon worden verbeterd. Het toevoegen van stollingsfactor VIIa, fosfolipiden, adenosine difosfaat en trombine zorgde niet voor een verbeterde bloedstolling. Behandeling van de PU met 'plasma glow discharge' (plasma glimontlading) gaf wel een betere bloedstolling. Deze behandeling wordt toegepast om biomaterialen, waaronder polyurethaan, meer hydrofiel te maken. Ook het verhogen van de PEG concentratie naar 55 wt% (gewichtsprocent), en daarmee de hydrofiliciteit van het materiaal, zorgde voor betere resultaten die vergelijkbaar waren met gelatine. In deze studie werden de beste resultaten behaald met collageen, bij welk materiaal de afname van de stroomsnelheid van het bloed significant sneller was dan bij PU.
Uit deze studie werd geconcludeerd dat het verhogen van de PEG concentratie in het PU schuim een veelbelovende aanpak is. Daarom is de effectiviteit en oplosbaarheid van dit materiaal verder onderzocht in de volgende hoofdstukken.
Omdat de bloedstolling in een testopstelling in het laboratorium (*in vitro*) niet volledig is na te bootsen, zijn er ook studies gedaan bij mensen en dieren. Deze studies worden *in vivo*

studies genoemd en in **hoofdstuk 3** is een dergelijke studie beschreven. Hierbij zijn 60 patiënten geïncludeerd waarbij een kies in de boven- en onderkaak moest worden verwijderd. Nadat de kiezen eruit gehaald waren, is het PU schuim in de ene wond en een collageen- of gelatinespons in de andere wond gedaan gedurende 2 minuten. Op deze manier kon het PU schuim met collageen en gelatine worden vergeleken in eenzelfde patiënt. Nadat het testmateriaal zich gedurende 2 minuten had kunnen volzuigen met bloed werd het uit de wond gehaald en afgedraaid in een centrifuge, zodat metingen konden worden gedaan aan het bloed uit de testmaterialen. In dit bloed zijn de concentraties van trombine-antitrombine III (TAT) complexen, fibrinogeen en tromboxaan B_2 (TxB_2) gemeten om informatie te krijgen over de mate van stolling in de verschillende testmaterialen. Deze concentraties werden ook bepaald in bloedmonsters die uit de wond werden opgezogen om een basiswaarde te krijgen. Uit de metingen bleek dat de concentraties van de TAT complexen en TxB_2 significant hoger waren, en de fibrinogeenconcentratie significant lager was dan de concentraties in het bloed dat rechtstreeks uit de wond was gezogen. Hieruit bleek dat er toegenomen bloedstolling was in de materialen bovenop de activatie van de bloedstolling in de wond door het lichaam zelf. Er werden geen significante verschillen gezien tussen de testmaterialen onderling. Uit deze resultaten werd geconcludeerd dat de hemostatische effectiviteit van PU schuim, gelijk is aan collageen en gelatine en daardoor een veelbelovend synthetisch alternatief is voor deze producten die op basis van dierlijke materialen vervaardigd zijn.

Ook in **hoofdstuk 4** is een *in vivo* studie beschreven. In deze studie werden verschillende hemostatica vergeleken met het PU schuim door ze te testen in een gestandaardiseerde stollingstest bij ratten. Ook werd PU getest waaraan de stollingsbevorderende stof chitosan was toegevoegd om te analyseren of de hemostatische effectiviteit hierdoor kon worden verbeterd. In totaal werden 80 ratten gebruikt voor deze studie, hierdoor konden 8 groepen met 10 ratten worden samengesteld. Bij de ratten werd aan het uiteinde van de staart een wond gemaakt nadat de staart op een testopstelling was gefixeerd. De wond werd vervolgens in de testopstelling met een constante en gelijke druk door één van de testmaterialen afgedrukt. Vervolgens werd de tijd gemeten tot de wond volledig gestold was zodat de gemiddelde stollingstijden met elkaar konden worden vergeleken.
De tijd tot volledige stolling was 23.9 minuten voor het PU schuim. Dit was niet significant korter of langer dan de stollingstijden van gelatine (23.6 min), collageen (28.2 min) en cellulose (26.9 min). Het PU schuim waaraan chitosan was toegevoegd liet de kortste gemiddelde stollingstijd zien (21.5 min) maar het verschil met de PU zonder chitosan was niet significant. Uit deze studie werd geconcludeerd dat de hemostatische effectiviteit van PU gelijk is aan de meest gebruikte hemostatica. Het toevoegen van de stollingsbevorderende stof chitosan liet een iets snellere gemiddelde stollingstijd zien, maar het verschil was niet significant.
In **hoofdstuk 5** is een studie beschreven naar de stollingsbevorderende eigenschappen van PU schuim waarbij dit werd vergeleken met collageen en gelatine. Ook de visco-elastische eigenschappen van de materialen werden geanalyseerd door de vervormbaarheid en elas-

ticiteit onder verschillende omstandigheden te meten. De stollingseigenschappen werden getest door het effect op de aggregatie (samenklontering) van bloedplaatjes in humaan bloed te meten en het effect op de stollingstijd van bloedplasma zonder bloedplaatjes te bepalen. Het vermogen van de materialen om druk uit te kunnen oefenen op de wond werd gekwantificeerd door de visco-elastische eigenschappen te bepalen onder droge en natte condities met behulp van een testopstelling.
Het lage aantal vrije bloedplaatjes in de bloedmonsters van PU schuim en collageen duiden erop dat onder invloed van deze materialen een groot gedeelte van de bloedplaatjes samenklonteren. Het aantal vrije bloedplaatjes in de bloedmonsters van gelatine verschilde niet significant van de bloedmonsters uit de controlegroep. De gemiddelde stollingstijden van de plasmamonsters die geïncubeerd waren met PU and collageen waren significant korter dan de stollingstijden van de controlemonsters zonder materiaal. Hieruit werd afgeleid dat PU en collageen ook zorgen voor initiatie van de stollingscascade. De gemiddelde stollingstijd van de plasmamonsters die geïncubeerd waren met gelatine was niet significant verschillend van de gemiddelde stollingstijd van de controlemonsters zonder materiaal. Analyse van de viscoelastische eigenschappen liet zien dat de vervorming onder natte condities van collageen en gelatine groter is dan de vervorming van PU schuim. Ook de mate waarin in het materiaal terugkeert naar zijn oorspronkelijke vorm nadat het is ingedrukt was groter voor PU dan voor collageen en gelatine.
Uit deze studie werd geconcludeerd dat PU schuim en collageen tot bloedstolling leiden doordat deze materialen zorgen voor aggregatie van bloedplaatjes en de stollingscascade activeren. Gelatine liet in deze studie geen verbetering zien van aggregatie van bloedplaatjes of activatie van de stollingscascade in vergelijking met de controlemonsters. De visco-elastische eigenschappen van PU schuim zijn gunstiger dan die van collageen en gelatine omdat het PU schuim zijn stevigheid behoudt terwijl collageen en gelatine hun mechanische eigenschappen verliezen. Hierdoor kan er met het PU schuim een betere druk op de wond worden uitgeoefend en is het in natte toestand beter te hanteren dan collageen en gelatine.

Voor een goed hemostaticum is het van belang dat het snel degradeert (oplost) in het menselijk lichaam en niet leidt tot ontstekingsreacties of ernstige vreemd-lichaam reacties. Daarom is een studie gedaan naar de degradatiesnelheid en -eigenschappen van PU schuim waarbij weer een vergelijk is gemaakt met collageen en gelatine. Deze studie is beschreven in **hoofdstuk 6**. De materialen werden subcutaan (onderhuids) geïmplanteerd in ratten en konijnen die op bepaalde tijdsintervallen tot drie jaar werden getermineerd. De nierfuncties van de dieren werden op deze tijdsintervallen gemeten om de mogelijke toxische effecten van PEG op de nieren te kunnen analyseren. Na terminatie van de dieren werden de materialen opgezocht en uitgenomen waarna ze werden onderzocht met een lichtmicroscoop.
Uit dit onderzoek bleek dat het patroon van degradatie gelijk was voor de verschillende materialen. De tijd tot volledige degradatie was echter aanzienlijk korter voor collageen en gelatine dan voor PU schuim. Het duurde 3 jaar na implantatie voordat het PU schuim niet meer werd

gezien terwijl gelatine en collageen al na respectievelijk 12 en 26 weken niet meer zichtbaar waren. Bij geen van de materialen werden ontstekingsreacties of ernstige vreemd-lichaam reacties gezien. De nierfuncties van de dieren bleven gedurende de onderzoeksperiode van drie jaar binnen de normaalwaarden en het PEG had dus in de gebruikte hoeveelheden geen toxische effecten op de nieren. Op basis van deze resultaten werd geconcludeerd dat PU schuim veilig gebruikt zou kunnen worden als hemostaticum. Het zou echter gunstiger zijn als PU schuim net zo snel zou degraderen als collageen en gelatine.

In **hoofdstuk 7** zijn de effecten op nabloedingen geëvalueerd van het doorgebruiken van anticoagulantia tijdens operatieve ingrepen in de mond. Voor deze studie werden 206 patiënten geïncludeerd waarvan er 103 geen anticoagulantia gebruikten en 103 wel anticoagulantia gebruikten. In de groep patiënten die wel anticoagulantia gebruikten waren er 71 die bloedplaatjesremmers gebruikten en 32 die vitamine K antagonisten gebruikten die aangrijpen op de stollingscascade. Patiënten werden behandeld volgens een richtlijn die is ontwikkeld aan het Academisch Centrum Tandheelkunde Amsterdam (ACTA). De operatieve ingrepen in de mond werden verdeeld in de volgende categorieën: chirurgische verwijdering van kiezen, niet-chirurgische verwijdering van kiezen, apexresecties (wortelpuntbehandelingen) en het plaatsen van implantaten. Alle patiënten kregen standaard postoperatieve zorg en de patiënten die vitamine K antagonisten gebruikten moesten daarnaast spoelen met tranexaminezuur mondspoeling. Het tranexaminezuur zorgt ervoor dat het bloedstolsel minder snel wordt afgebroken in de mond.
Geen van patiënten kreeg een ernstige nabloeding waarvoor medisch ingrijpen noodzakelijk was. In de groep met patiënten die anticoagulantia gebruikten waren er zeven patiënten (6.8%) die een lichte nabloeding hadden na de ingreep. In de controlegroep met patiënten die geen anticoagulantia gebruikten kregen er twee (1.9%) een lichte nabloeding. Deze lichte nabloedingen konden door de patiënten zelf gestelpt worden door een gaasje op de wond te drukken. Uit deze studie werd geconcludeerd dat het veilig is om operatieve ingrepen in de mond te doen bij patiënten die anticoagulantia gebruiken, mits de ACTA richtlijn gevolgd wordt.

In **hoofdstuk 8** zijn de belangrijkste onderzoeksresultaten besproken, waarbij ook aanbevelingen voor toekomstig onderzoek zijn gedaan. Uit dit onderzoek is geconcludeerd dat de hemostatische effectiviteit van polyurethaanschuim met 55 wt% polyethyleenglycol gelijk is aan de hemostatica op basis van collageen en gelatine. Het toevoegen van stollingsbevorderende stoffen aan het polyurethaanschuim heeft in de beschreven onderzoeken niet geleid tot een significant betere stolling. De visco-elastische eigenschappen van polyurethaan zijn gunstiger dan die van gelatine en collageen, waardoor er enige compressie op een wond kan worden uitgeoefend en het in natte toestand goed te hanteren is. De tijd tot volledige degradatie van het PU schuim is langer dan de degradatietijden van collageen en gelatine. Verder wordt geconcludeerd dat het veilig is om operatieve ingrepen in de mond te doen bij patiënten die anticoagulantia gebruiken, mits aan bepaalde voorwaarden is voldaan.

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Curriculum Vitae

Ferdinand Isaäk Broekema was born on March 12th 1983 in Amsterdam, the Netherlands. After finishing secondary school (gymnasium; Bornego College, Heerenveen) in 2001 he started his study Medicine at the University of Groningen. After obtaining his medical degree (MD) in 2008, he worked at the emergency department of the Delfzicht Hospital in Delfzijl. In 2009 he started his PhD project at the department of Oral and Maxillofacial Surgery of the University Medical Center Groningen (UMCG). In the same year, he also began with his Dentistry study at the University of Groningen. From 2013 he worked as a dentist in a private practice in Beilen. In March 2014 he started with his residency at the Department of Oral and Maxillofacial Surgery in the UMCG (head: Prof. dr. F.K.L. Spijkervet).
Ferdinand is married to Joline Broekema-de Jager. Together they have a son, Aaron, who was born on April 27th 2013.

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